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THE EFFECT OF DIETARY PROTEIN ON THE CATABOLIC INFLUENCE OF PROGESTERONE*

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ABSTRACT

In 6 comparative studies carried out on 5 healthy subjects, it was shown that the catabolic influence of administered progesterone could be enhanced by substantial dietary protein increments or suppressed by the restriction of protein intake. These effects were observed if the protein content of a diet was increased without alteration in carbohydrate or fat and also when protein was added at the expense of carbohydrate thus maintaining a constant caloric value in comparative diets. Since the digestion and absorption of dietary protein is known to increase the size of the metabolic pool of amino acids, it was concluded that under the conditions of these experiments progesterone was a more effective catabolic agent when this pool was large. It was accordingly suggested that progesterone enhanced the net rate of protein catabolism primarily by accelerating the utilization of amino acids by the liver.

IN MAN progesterone enhances the net rate of protein catabolism(1–3). The moderately intense catabolic process induced by physiologic quantities of the hormone is not mediated by pituitary, thyroid or adrenocortical secretions; nor is the presence of estrogen required (4). Early descriptions of the catabolic process provided some indications that it could be distinguished from the enhancement of protein catabolism induced by

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excesses of thyroid and adrenocortical secretions (2). However, beyond deductions derived from these comparisons, little is known concerning the means by which this influence on protein metabolism is brought about. A catabolic effect was not anticipated, and its significance and importance, especially during pregnancy, is obscure. The first efforts to gain greater understanding of the process are those to be reported here.

The intensity of nitrogen losses induced by repeated courses of progesterone in one subject receiving the same constant diet have been quite reproducible (2,4,5). It was accordingly felt that some hint of the mechanism involved might be obtained by determining the effect of alterations in the diet on the catabolic process. Comparisons were made between the urinary nitrogen increments induced by standard courses of progesterone given to subjects at different levels of dietary protein, usually with little or no caloric change. It was anticipated that this would provide a test of the influence of the quantity of readily available amino acids on the rate of their metabolism under the influence of progesterone.

METHODS

The subjects, 4 normal young men and an ovariectomized woman aged 51 years, were hospitalized in the metabolic study unit. Sporadic bursts of physical activity were prohibited, but the usual activities of a medical student or floor nurse were allowed. Each subject was given an appropriate calorically adequate constant diet, and control observations were begun after an interval for metabolic equilibration. Progesterone in a dosage of 50 mg. per day was then given for eight or ten consecutive days. When urinary nitrogen excretion had returned to the baseline level following treatment, the diet was changed according to plan. When a new control level of nitrogen excretion had been assumed, the identical course of progesterone was repeated. In some cases the higher protein diet was given first; in others the study was begun with a low protein intake. In each experiment the magnitude of the nitrogen loss was calculated by totaling the difference between the control baseline and the daily urinary nitrogen output during the course of progesterone treatment.

Urinary and fecal nitrogen were determined by a semi-microKejldahl analysis with steam distillation (6), inorganic phosphate by a modification of the Taussky and Shorr procedure (7), creatine and creatinine by a modification of Folin's method as described by Peters and Van Slyke (8), and potassium by the Perkin-Elmer flame photometer after loading specimens and standards with sodium chloride.

RESULTS

In all of the comparative studies the catabolic effect of progesterone, as indicated by the elevation in urinary nitrogen, was significantly greater during the period of higher protein diets. In Subject R.Mc. (Fig. 1) the eight-day urinary nitrogen increment induced by progesterone totaled 14 gm. when his diet contained 37 gm. of protein. Following the addition of 86 gm. of protein, maintaining carbohydrate and fat at a constant level, the same course of progesterone induced a urinary nitrogen elevation

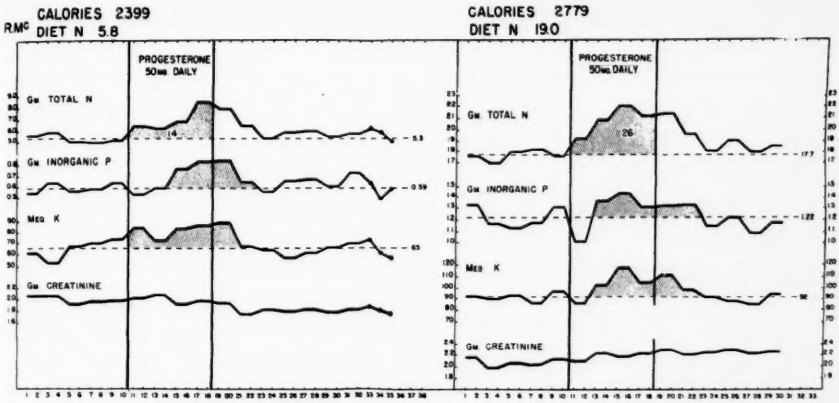

FIG. 1. The effect of progesterone on urinary nitrogen, inorganic phosphorus, potassium and creatinine in the normal young man, R.Mc. During the first 8-day course of progesterone his diet consisted of 317 gm. of carbohydrate, 37 gm. of protein (N, 5.8 gm.) and 109 gm. of fat; calories, 2399. The second diet contained 317 gm. of carbohydrate, 123 gm. of protein (N, 19.0 gm.) and 109 gm. of fat; calories, 2741. Vertical lines delimit courses of therapy; shaded areas indicate significant shifts from the control baselines (the broken horizontal lines).

which totaled 26 gm. In a similar study in Subject J.M. (Fig. 2), 71 gm. of protein was added with no change in the carbohydrate and fat content. The nitrogen loss induced during the lower protein program was 21 gm., whereas with the 120-gm. protein diet the elevation totaled 34 gm. A new,

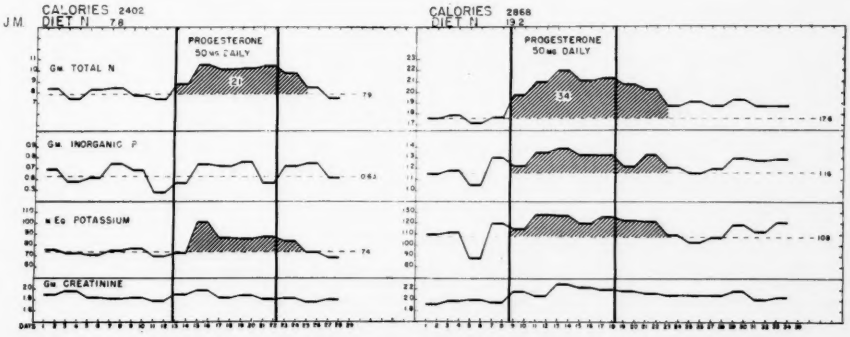

FIG. 2. The effect of progesterone in urinary nitrogen, inorganic phosphorus, potassium and creatinine in the normal young man, J.M. The first diet consisted of 358 gm. of carbohydrate, 49 gm. of protein (N, 7.8 gm.) and 86 gm. of fat; calories, 2402. The second diet contained 358 gm. of carbohydrate, 120 gm. of protein (N, 19.2 gm.) and 86 gm. of fat; calories, 2686. Vertical lines delimit courses of treatment; shaded areas indicate significant shifts from the control averages (the broken horizontal lines).

slightly higher baseline which was assumed following the latter course of progesterone might place in question the calculated nitrogen elevation of 34 gm. Accordingly, the course of progesterone was repeated with essentially the same diet (Fig. 3), and in this instance the calculated loss was also greater than that induced during the period of lower protein diet (28 gm.).

When the protein content of diets was lowered to the point of frank protein restriction, the catabolic response was slight indeed, and in the postmenopausal woman, B.P. (Fig. 4), urinary nitrogen excretion was absolutely uninfluenced when she consumed only 24 gm. of protein per day.

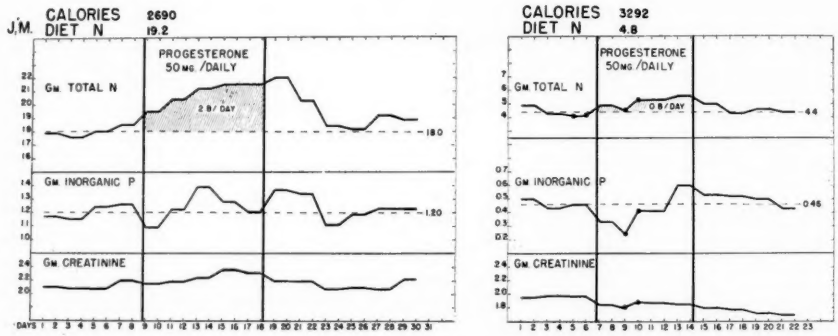

FIG. 3. The effect of progesterone on the urinary excretion of nitrogen, inorganic phosphorus and creatinine in the normal young man, J.M. The first diet contained 359 gm. of carbohydrate, 120 gm. of protein (N, 19.2 gm.) and 86 gm. of fat; calories, 2690. The second contained 214 gm. of carbohydrate, 30 gm. of protein (N, 4.8 gm.) and 124 gm. of fat; calories, 3292. Vertical lines delimit days of treatment with progesterone. Shaded areas indicate shifts from the control averages (horizontal broken lines) thought to be significant.

When her dietary protein was increased to 106 gm. with little change in carbohydrate, a modest nitrogen increase which averaged 1 gm. per day was induced. Similarly in Subject J.M. (Fig. 3), when the dietary protein was lowered to 30 gm., the total nitrogen loss was a minimal 6.4 gm.

The possibility that the anabolic influence of a caloric surfeit would suppress the nitrogen-wasting effect of progesterone had also to be considered. In Subject J.M. (Fig. 3) the 30-gm. protein diet contained about 600 calories more than the two 120-gm. protein diets employed in the same subject. This caloric addition may have been a factor in suppressing the catabolic response to progesterone, but it was almost certainly not the dominant influence. In both Subjects R.MC. (Fig. 1) and B.P. (Fig. 4) and the first study in Subject J.M. (Fig. 2) the higher protein diets also con-

tained 200 to 350 more calories per day, and still the catabolic influence of progesterone was greater than during the lower protein and calorie programs.

A diminution in dietary protein content without a significant caloric modification was also associated with the induction of a smaller urinary nitrogen elevation. This was well illustrated in the study on Subject D.C. (Fig. 5) in whom the ten-day course of progesterone induced almost identical catabolic processes (32-gm. losses) during the two periods of 120-gm. protein diets. When the protein intake was just 53 gm. per day and the total calories differed very little, the ten-day nitrogen increment totaled 23 gm. Similar observations were made in Subject S.E. (Fig. 6) in whom the

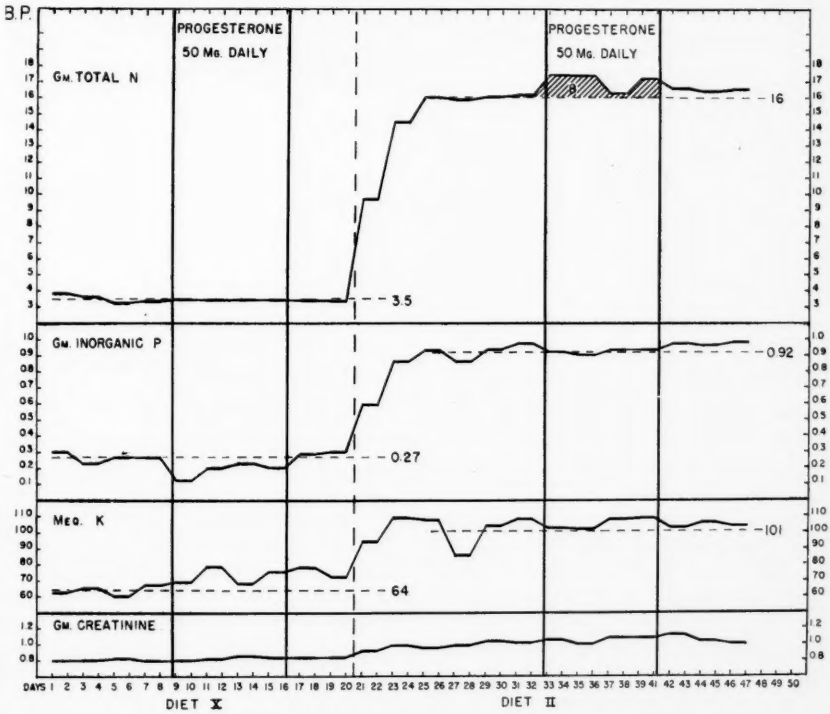

FIG. 4. The effect of progesterone on the urinary excretion of nitrogen, inorganic phosphorus, potassium and creatinine in the ovariectomized female, B.P. Diet X contained 301 gm. of carbohydrate, 24 gm. of protein (N, 3.84 gm) and 87 gm. of fat; 2085 calories. Diet II consisted of 280 gm. of carbohydrate, 106 gm. of protein (N, 17 gm.) and 88 gm. of fat; 2336 calories. The broken vertical line denotes diet change. The heavy vertical lines delimit courses of treatment. The shaded areas indicate a significant deviation from the control baselines (broken horizontal lines).

nitrogen loss induced during a 150-gm. protein diet period was 32 gm., and with the reduction of dietary protein to 51 gm. without a notable caloric change the nitrogen elevation totaled just 12 gm. In this subject fecal nitrogen excretion was unaffected by changes in dietary protein or treatment with progesterone.

Although the total urinary nitrogen elevation induced by progesterone was always distinctly greater when the subjects consumed high protein meals, the urinary phosphorus and potassium responses to the progesterone could not be so clearly related to the protein intake. In Subject D.C. (Fig

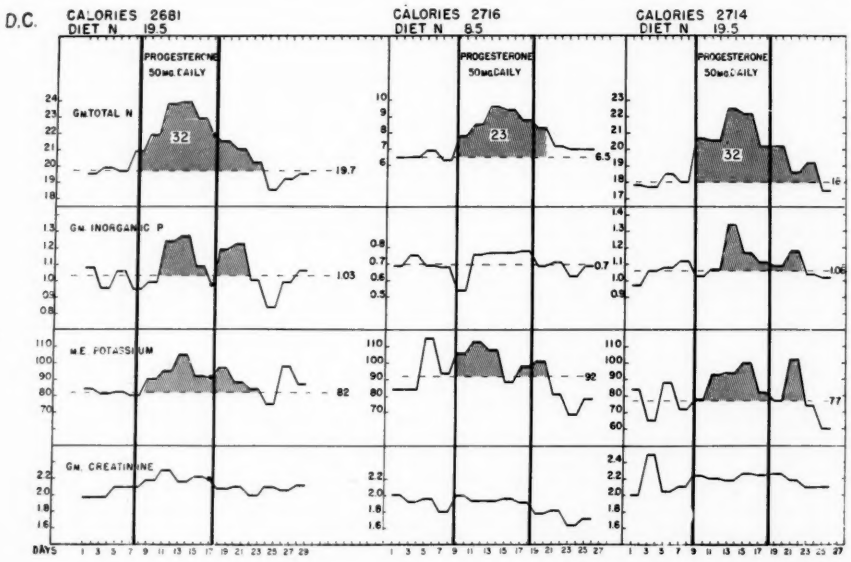

FIG 5. The effect of progesterone on urinary nitrogen, inorganic phosphorus, potassium, and creatinine in the normal young man, D.C. The heavy vertical lines delimit courses of treatment. Shaded areas indicate significant shifts from the control averages (horizontal broken lines). The first diet contained 195 gm. of carbohydrate, 122 gm. of protein (N, 19.5 gm.) and 157 gm. of fat; 2681 calories. The second diet contained 401 gm. of carbohydrate, 53 gm. of protein (N, 8.5 gm.) and 100 gm. of fat; 2716 calories. The third diet contained 199 gm. of carbohydrate, 122 gm. of protein (N, 19.5 gm.) and 159 gm. of fat; 2714 calories.

5) a somewhat irregular but distinct rise in inorganic phosphorus accompanied the nitrogen elevation during the two courses given during high protein feedings, but only a questionable increase was apparent when the catabolic response was less during a period of 58-gm. protein diet. Potassium losses induced were much the same in all the studies in Subject D.C.

In the 4 studies in Subject J.M. (Figs. 2 and 3) the rise in inorganic phosphorus was most distinct during the first period of high protein diet (Fig. 2). It was equivocal during the second one, as well as during the period of moderate (49-gm.) protein diet; no phosphate loss was evident when he consumed just 30 gm. of protein per day. The increase in urinary potassium was slightly greater when progesterone was given during the high protein regimen than during the moderate protein regimen. In Subject R.Mc. (Fig. 1), inorganic phosphorus and potassium excretions rose to about the same extent in the two studies; and in Subject B.P. the excre-

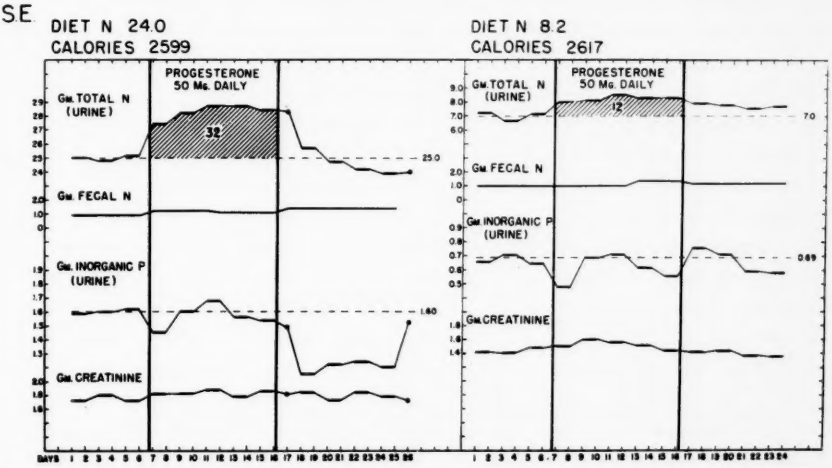

FIG. 6. The effect of progesterone on urinary nitrogen, inorganic phosphorus and creatinine and fecal nitrogen in the normal young man, S.E. The initial diet contained 259 gm. of carbohydrate, 150 gm. of protein (N, 24.0 gm.) and 107 gm. of fat; calories, 2599. The second diet consisted of 439 gm. of carbohydrate, 51 gm. of protein (N, 8.2 gm.) and 63 gm. of fat; calories, 2617. The vertical lines delimit treatment periods. The shaded areas signify significant deviation from the control averages (horizontal broken lines).

tion of neither of these constituents was enhanced on either occasion by administration of progesterone. There was also no loss of inorganic phosphorus during either of the treatment courses in Subject S.E. (Fig. 6).

DISCUSSION

The enhancement of the catabolic influence of progesterone by the addition of dietary protein, or the curtailment of the process by sharp protein restriction has been observed in 5 normal subjects. Other dietary factors such as a moderate caloric addition or the relative amounts of carbohydrate and fat did not appear to be influential in this regard. The

anabolic effect of a supermaintenance diet might be expected to counteract the catabolic force of the hormone (9), and in one study (J.M., Fig. 3) the additional 600 calories in the low protein period may have played a role in the suppression of the catabolic process. However, in the other studies when up to 350 calories were added with a higher quantity of dietary protein, the nitrogen-sparing influence of a caloric surfeit was not apparent.

In medical circles it is often thought that a high dietary content of good quality protein will protect the subject from catabolic influences. As a matter of fact, careful studies indicate that with an adequate quantity of dietary protein a caloric excess is more effective (9). The catabolic influence of an excess of adrenocorticoids is readily counteracted by a caloric excess (10), but a substantial change in protein intake without any change in calories has no detectable influence on this hormonally induced catabolic process (11). It was not, however, expected that the nitrogen losses induced by progesterone would be enhanced by substantial additions of dietary protein.

A high protein meal results in an enlargement of the metabolic pool of amino acids. The tissues of the body draw amino acids from this pool for protein anabolism, glucose, glycogen and fat synthesis and direct energy production (12). The results of the studies reported here may be interpreted as indicating that when the metabolic pool of amino acids is enlarged, progesterone favors to an even greater extent than otherwise the utilization of amino acids for energy to support metabolic activity. It is likely that much of the amino acids metabolized under the influence of progesterone is exogenous. However, dietary protein does not account for all of the increment in protein catabolism. The negative nitrogen balance induced was always greater when progesterone acted at a time when the metabolically available pool of amino acids was larger.

If progesterone acted directly on peripheral tissues as is apparently the case with the adrenocorticoids (13), one could anticipate that the intensity of the catabolic process would be lessened or unaffected by the addition of dietary protein. The enhancement of protein catabolism in this circumstance thus suggests a metabolically central action of the hormone in accelerating utilization of amino acid. Accordingly, it seems appropriate to propose that progesterone enhances the hepatic utilization of amino acids so effectively that the rate of tissue protein catabolism elsewhere is secondarily enhanced. This hypothesis is supported by the finding that the level of plasma amino acid nitrogen is lowered when progesterone acts catabolically (14).

Some observers may be troubled by the relatively poor correlation between total urinary nitrogen excretion and the urinary output of inorganic

phosphorus and potassium. Potassium and phosphate are intimately associated with tissue protein, and are doubtless liberated from cells with accelerated tissue catabolism. In metabolic studies of this sort their excretion in the urine may parallel shifts in nitrogen excretion. However, this is not always the case under acute conditions. A poor correlation has not only been noted here, but also in earlier studies with progesterone (2, 4, 5).

It must be appreciated that both phosphate and potassium are involved in metabolic processes other than protein turnover, and their excretion in urine may be affected by these other metabolic activities as well. For example, inhibition of aldosterone's sodium-retaining action by progesterone (15) may also extend to inhibition of the kaliuretic action. This influence would oppose the kaliuresis expected with enhanced protein catabolism. The fact that much of the amino acid utilized is probably exogenous and thus not bound to potassium and phosphate may contribute further to the lack of parallel movement of these substances and nitrogen in such relatively short studies. If much more protracted tissue wastage were to be induced, other factors would perhaps be less important, and a closer approximation of nitrogen, inorganic phosphorus and potassium loss to their relative concentrations in tissue protein would probably obtain (16).

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THE EFFECT OF PROGESTERONE ON AMINO ACID METABOLISM*

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ABSTRACT

The concentration of plasma alpha amino acid nitrogen was significantly lowered by the intramuscular administration of 50 mg. per day of progesterone in normal volunteers. This suppression (1.3–1.7 mg. per 100 ml.) was more pronounced during the peak rise in amino acid nitrogen concentration which followed a standard high protein meal than when comparisons were made in fasting subjects or four hours after a low protein meal. These effects were observed as soon as seven hours after the first injection of progesterone. During three-day fasts, the amino acid nitrogen concentrations averaged 0.5–0.7 mg. per 100 ml. lower when daily injections of progesterone were given. The plasma glucose concentrations were unaffected by progesterone treatment. The fact that the concentration of plasma alpha amino acid nitrogen is promptly lowered without an increase in urinary amino acid excretion strongly supports the previous suggestion that the primary action of progesterone in enhancing the net rate of protein catabolism is exerted on the utilization of amino acids by the liver. The net increase in protein catabolism would thus be secondary to the lowered plasma concentration of amino acids. Whether tissue protein anabolism is inhibited or catabolic processes are accelerated remains to be determined.

PREVIOUS studies have described the enhancement of protein catabolism induced by progesterone in man, and have established that this is a manifestation of progesterone action not mediated by other hormones (1–3). Certain of the metabolic features readily distinguish it from other hormonally induced catabolic processes in man, such as those due to thyroid and adrenocortical secretions: *1)* Although protein catabolism may be enhanced by as little as 15 mg. of progesterone per day, a maximal response of only moderate intensity is induced by approximately 50 mg. of progesterone per day, a dosage within the physiologic range (4); substantially greater catabolic effects can be induced by excesses of thyroid or adreno-

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cortical hormones (5, 6); *2)* Uricosuria is not prominent as in the case of adrenocortical secretions (2, 7); *3)* Urinary amino acid nitrogen excretion is either unaffected or declines slightly during progesterone treatment, but rises sharply when protein catabolism is enhanced by thyroid or adrenocortical secretions (2, 8); *4)* The intensity of the catabolic response to progesterone is increased significantly by a high intake of dietary protein (9). At least with respect to the corticoids, the amount of dietary protein alone does not seem to influence the magnitude of the catabolic effect (10).

The anabolic influence of progesterone on sex-linked and related tissues, endometrium, breast parenchyma, sebaceous glands and the levator ani muscle of rats (11–14) would suggest that the hormone might also be generally anabolic, as is the case with androgen and estrogen (15). The surprising catabolic influence might thus be expected to have an unusual explanation. The enhancement of the induced catabolic process by a high protein intake and the failure of progesterone to induce a heightened amino aciduria provided a clue. Accordingly, the effect of progesterone on the concentration of plasma amino acid nitrogen was studied.

METHODS

The subjects were normal male volunteers who lived in the metabolic unit and carried on their usual activities as students. They consumed constant adequate diets throughout. Their protein intakes were relatively high, with deliberate efforts to concentrate much of the dietary protein at the noon meal. Physical activities were reasonably constant from day to day.

It soon became apparent, as had been shown by Van Slyke and Meyer (16), that high protein-containing meals were followed by significant elevations in the concentration of amino acid nitrogen. About two to three hours after high protein lunches, the peak elevations (2–3 mg. per 100 ml.) of plasma alpha amino acid nitrogen occurred. For each subject a schedule of blood lettings was then established, with at least three specimens being drawn during this postprandial elevation period. In each of several subjects the concentration of plasma amino acid nitrogen throughout a number of control days was then compared with values obtained while the subject was receiving progesterone in a dosage of 50 mg. per day. Periods of treatment were spaced in a more or less random fashion throughout a two-month or three-month interval. Blood specimens were drawn on various days of a treatment course and on scattered days before and after treatment with progesterone. Since the catabolic effect of the hormone may persist for two to six days after its discontinuance, values secured within thirty-six hours of the last intramuscular dosage were assumed to be influenced by treatment. Urinary nitrogen excretion (data not shown here) was followed, to ascertain that control plasma determinations were carried out at the same level of protein catabolism and to provide evidence that progesterone was enhancing nitrogen excretion as expected. In 1 subject, glucose concentrations were measured in the same heparinized blood samples used for amino acid analysis.

In 3 subjects the concentration of plasma alpha amino acid nitrogen and the urinary excretion of nitrogen were compared through two 3-day fasts (water was permitted without restriction). Progesterone in a dosage of 50 mg. per day was given through one of the fasts in each subject. The prefasting diets were identical for each of the two fasts. In 2 of

these volunteers the levels of urinary nitrogen excretion were practically the same prior to each of the fasts. In the third, however, the control urinary nitrogen level was 1.0 gm. higher before the initial control fast.

Plasma alpha amino acid nitrogen levels were determined by the method of Russell (17, 18), and glucose by the Shaffer-Hartman procedure (19). Total urinary nitrogen and creatinine were determined as previously described (9).

RESULTS

In every subject the plasma alpha amino acid nitrogen levels were significantly lowered as a result of intramuscularly administered progesterone. As anticipated, protein catabolism was enhanced by each protracted course of progesterone. However, when only scattered single injections of the hormone were given as in Subject V.B. (Fig. 3) a significant elevation in the 24-hour urinary nitrogen output could not be defined, because in

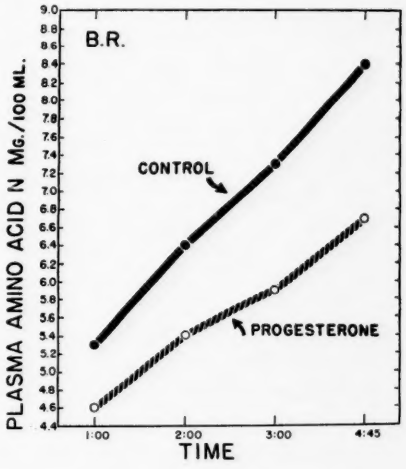

FIG. 1. Concentration of plasma amino acid nitrogen following a lunch containing 60.7 gm. of protein in the normal man, B.R. The solid line depicts the average of values obtained on 11 control days at the times noted. The broken line indicates the averages secured on eight days during which 50 mg. per day of progesterone had been given. Standard errors of the mean values at each time are as follows: Controls—1:00 p.m., 0.05; 2:00 p.m., 0.15; 3:30 p.m., 0.17; 4:45 p.m., 0.1. Progesterone—1:00 p.m., 0.1; 2:00 p.m., 0.16; 3:30 p.m., 0.22; 4:45 p.m., 0.20.

the usual pattern of rise in urinary nitrogen during a course of progesterone the first-day effect is usually minimal or not detectable (2).

In the normal man, B.R. (Fig. 1), the control average concentration of plasma amino acid nitrogen rose progressively from an average of 5.3 mg. per 100 ml. just before lunch to a maximum of 8.4 mg. per 100 ml. three and three quarter hours later. When progesterone had been administered, the pre-lunch average was 4.6 mg. per 100 ml., and successive values through the afternoon reached a peak of only 6.7 mg. per 100 ml. The pre-feeding level during progesterone treatment thus averaged 0.7 mg. per 100 ml. below the control level, and the difference between treatment and control averages widened postprandially to 1.7 mg. per 100 ml. This highly significant departure from the control curve was indicated by

the aggregate of treatment values despite the fact that the concentrations of amino acid nitrogen on one of the treatment days included in the average were 5.2, 6.3, 6.9 and 8.0 mg. per 100 ml.—values virtually identical with those of the control curve. These results were the only ones obtained in this subject on the first day of treatment, thus suggesting that in him the effect of progesterone on amino acid metabolism was not especially prompt. Urinary nitrogen excretion also failed to rise the first two days of this course of progesterone, although the usual catabolic process was eventually manifested.

In Subject N.L. (Fig. 2), the plasma amino acid nitrogen level on control days rose following lunch from a fasting average of 5.4 mg. to a

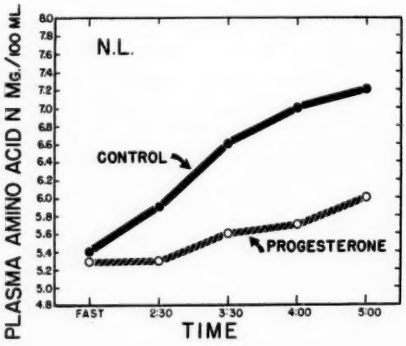

FIG. 2. Concentration of plasma amino acid nitrogen following a lunch containing 64 gm. of protein in the normal man, N.L. The control curve depicts the average of values obtained on nine scattered days. The values denoted by the broken line are averages for five days under treatment with 50 mg. of progesterone per day. Standard errors of the mean values are as follows: Control—fasting, 0.08; 2:30 p.m., 0.08; 3:30 p.m., 0.12; 4:00 p.m., 0.08; 5:00 p.m., 0.08. Progesterone—fasting, 0.14; 2:30 p.m., 0.25; 3:30 p.m., 0.26; 4:00 p.m., 0.18; 5:00 p.m., 0.1.

maximum of 7.2 mg. per 100 ml. During the five days on which progesterone was given, the fasting values (5.3 mg. per 100 ml., average) did not differ significantly, but the postprandial rise was slower and smaller. The 5:00 p.m. treatment values averaged 1.2 mg. lower than the controls. On three of the five treatment days, progesterone had not been given the prior day. In these blood samples which were drawn from the fasting subjects very shortly after the first intramuscular administration of progesterone, the concentrations were higher (average, 5.5 mg. per 100 ml.) than the levels of 4.8 and 5.0 mg. per 100 ml. observed when the experimental day was not the first in a series of injections. In this subject, however, the postprandial values on the initial days of treatment were distinctly lower than the controls, although slightly higher than the concentrations secured after several days under the influence of progesterone. In Subject V.B. (Fig. 3), progesterone was never given on consecutive days, and the amino acid concentrations during treatment could only be influenced by the progesterone given the morning of the blood lettings. Again, the plasma alpha amino acid nitrogen levels following the high protein lunch were significantly lowered by progesterone.

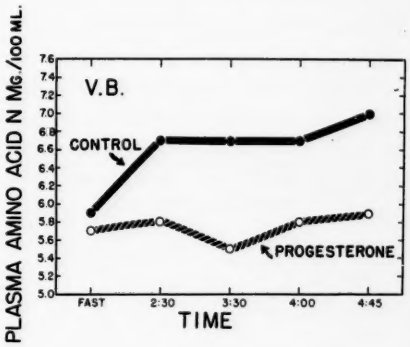

FIG. 3. Concentration of plasma amino acid nitrogen following a lunch containing 72.4 gm. of protein in the normal man, V.B. The control curve represents averages of values obtained on eight days at the times noted. The treatment averages (broken lines) were obtained on four widely separated days. Progesterone in a dosage of 50 mg. per day was given only on the day of blood letting. Standard errors of mean values are as follows: Control—fasting, 0.1; 2:30 p.m., 0.18; 3:30 p.m., 0.21; 4:00 p.m., 0.2; 4:45 p.m., 0.1. Progesterone—fasting, 0.14; 2:30 p.m., 0.18; 3:30 p.m., 0.17; 4:00 p.m., 0.18; 4:45 p.m., 0.15.

In Subject B.M. (Fig. 4), the effects of oral and intramuscular administration of progesterone in the same dosage were compared. As in the other subjects, the concentration of amino acid nitrogen was lowered by progesterone given intramuscularly, but the same preparation given orally had no detectable influence on amino acid metabolism, was not catabolic, and did not elevate the basal body temperature.

The suppression of plasma amino acid nitrogen levels was thus observed as soon as seven hours after the first intramuscular injection of progesterone in 2 subjects, but in another young man progesterone probably had no effect on the plasma amino acid concentration during a first day of treatment. A somewhat greater suppression of the plasma alpha amino acid nitrogen level was induced following a high protein meal (1.3–1.7 mg. per 100 ml.) than after a low protein meal or an overnight fast (0–1.0 mg. per 100 ml.).

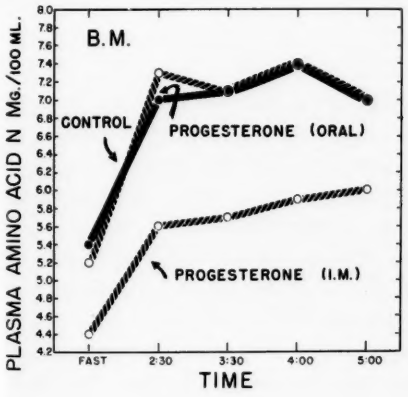

FIG. 4. Concentration of plasma amino acid nitrogen following a lunch containing 64.5 gm. of protein in the normal man, B.M. The control curve (solid line) represents the average of values secured on six days at the times noted. The upper broken line depicts the average values during two days of treatment with progesterone orally (50 mg. per day), and the lower one the averages during two days of treatment with progesterone intramuscularly (50 mg. per day). Standard errors of the mean control values are as follows: Fasting, 0.1; 2:30 p.m., 0.16; 3:30 p.m., 0.21; 4:00 p.m., 0.21; 5:00 p.m., 0.26.

Progesterone also lowered the concentration of alpha amino acid nitrogen during more protracted fasts. In the 3 normal men who fasted for three days on two separate occasions the plasma amino acid nitrogen values averaged 0.5, 0.5 and 0.7 mg. per 100 ml. lower respectively throughout the fasts in which progesterone was administered (Table 1). In 2 of the 3 subjects, the fasting urinary nitrogen excretion was higher (1.4 and 0.8 gm. per day) when progesterone was given during the fast; in the third

TABLE 1. THE EFFECT OF PROGESTERONE ON THE CONCENTRATION OF PLASMA AMINO ACID NITROGEN DURING THREE-DAY FASTS IN 3 NORMAL YOUNG MEN

Subject	Day	Time	Control (fasting) amino acid N (mg./100 ml.)	Progesterone treatment (fasting) amino acid N (mg./100 ml.)
B.M.	1	1:00 p.m.	5.0	4.7
		4:00 p.m.	5.2	4.6
	2	1:00 p.m.	5.3	4.4
		4:00 p.m.	5.2	4.3
	3	1:00 p.m.	5.5	4.6
		4:00 p.m.	5.4	4.7
		Average	5.3	4.6
N.L.	1	1:00 p.m.	5.3	5.3
		4:30 p.m.	5.1	4.8
	2	1:00 p.m.	5.5	4.8
		4:30 p.m.	5.3	5.0
	3	1:00 p.m.	5.5	4.7
		4:30 p.m.	5.7	4.8
		Average	5.4	4.9
B.R.	1	8:30 a.m.	5.2	5.1
		1:00 p.m.	4.7	4.4
	2	8:30 a.m.	5.3	4.7
		1:00 p.m.	5.7	4.8
	3	8:30 a.m.	5.6	5.3
		1:00 p.m.	5.8	4.9
		Average	5.4	4.9

man the fasting urinary nitrogen levels were practically identical but this was the subject in whom the control urinary nitrogen level was 0.9 gm. lower prior to the fast during which progesterone was given. Thus it is probable that the rate of protein catabolism during the three-day fast was also enhanced by administration of progesterone.

In Subject B.R. (Fig. 5) the concentration of venous blood glucose, determined at the same time as that of alpha amino acid nitrogen, rose as expected after a meal containing 71.0 gm. of carbohydrate. As can be seen, however, the glucose tolerance curves were virtually identical during progesterone treatment and the control days following the luncheon meal.

DISCUSSION

On theoretical grounds it may be assumed that hormones enhance the net rate of protein catabolism by either direct action on peripheral tissues or indirectly as a result of the acceleration of amino acid utilization by the liver. Adrenocortical and thyroid hormones, the potentially catabolic secretions which have been most exhaustively studied in man, apparently act directly on peripheral tissues. This mechanism is reflected in the elevated titers of plasma amino acid nitrogen which accompany their enhancement of protein catabolism and the simultaneous rise in urinary amino acids as well as urea (8, 20–22). Presumably when the greater quantity of amino acids made available by tissues reaches the liver, the

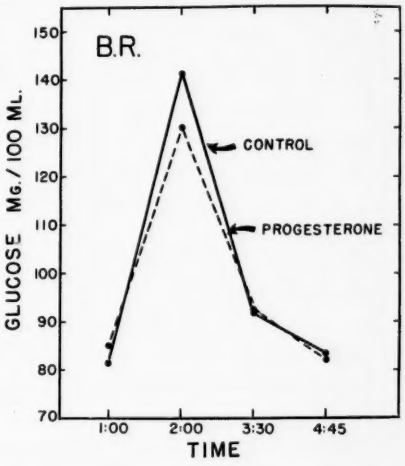

FIG. 5. The effect of progesterone on the blood glucose concentration in the same blood specimens used for amino acid determinations in the normal man, B.R. (Fig. 1). The solid line denotes the averages of values on control days; the broken line indicates averages on progesterone treatment days. Standard errors of mean values are as follows: Control—1:00 p.m., 1.0; 2:00 p.m., 5.6; 3:30 p.m., 1.5; 4:45 p.m., 1.9. Progesterone—1:00 p.m., 1.5; 2:00 p.m., 7.0; 3:30 p.m., 1.8; 4:45 p.m., 1.5.

total utilized is greater, and more urea is formed and excreted in the urine.

The fact that progesterone has a quite different effect on amino acid metabolism led us to the second mechanism noted as the explanation for its catabolic influence. If it acted directly on peripheral tissues, a rise in amino acid levels in venous plasma would not necessarily be expected. However, a significant decline such as that noted here cannot be reconciled with a direct enhancement of the rate of protein breakdown in tissues.

When progesterone enhances protein catabolism, the prompt decline in plasma amino acid concentration must reflect a primary effect on the utilization of amino acids by the liver. Urinary amino acid nitrogen is unaffected or even declines slightly (2). The plasma amino acid nitrogen titer is lowered prior to the initiation of a detectable rise in urinary nitrogen, and certainly well in advance of the maximal catabolic response. It is

accordingly suggested that the accelerated tissue protein catabolism induced by progesterone is the result of the lowered plasma amino acid concentration. Whether synthetic processes are retarded, or catabolic activity is accelerated, remains to be determined.

The enhancement of the catabolic influence of progesterone by substantial additions of protein to the diet had offered the first hint that the progesterone effect might be mediated by way of the liver (9). This effect of dietary protein on the magnitude of the catabolic process is consistent with the amino acid studies reported here. Indeed the influence of progesterone on amino acid nitrogen was more pronounced during the postprandial rise in amino acid concentration which uniformly followed a high protein meal than it was when the subjects were fasting.

A similar explanation has been offered for the catabolic influence of glucagon in rats, in which the enhanced protein expenditure induced is also accompanied by a decline in the level of plasma amino acid nitrogen (23). It appears from the results recorded here that such an effect on amino acid metabolism is not necessarily accompanied by any tendency toward hyperglycemia. An earlier study had also failed to demonstrate any influence of progesterone on carbohydrate metabolism in a diabetic subject (4).

The usefulness of this type of catabolic process to the organism, especially during pregnancy, is a mystery. Perhaps it represents a means of counteracting the anabolic influence of estrogen. A lowered titer of plasma amino acid nitrogen has been reported during pregnancy and it was proposed that this reflected the utilization of amino acids by the embryo and fetus (24). It can now be suggested that this represents the metabolic influence of the large amount of progesterone secreted at this time.

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6-CHLORO,6-DEHYDRO,17α-ACETOXYPROGESTERONE: ITS POSSIBLE ACTION AS AN ALDOSTERONE ANTAGONIST

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ABSTRACT

6-Chloro,6-dehydro,17α-acetoxyprogesterone (RS-1280) is a potent progestational agent, hence it was studied to determine whether or not it was an aldosterone antagonist. The study was carried out in 5 patients with cirrhosis of the liver and ascites, under constant metabolic conditions, with a previous examination of the renal capacity of each patient to excrete water, sodium and potassium under basal conditions and also when benzothiadiazine and mercurial diuretics were administered. In each case, RS-1280 was administered and its effect compared with that of a known aldosterone antagonist (spironolactone). By this means it was demonstrated that RS-1280 has no anti-aldosterone action, although it has a natriuretic effect in cirrhotic patients with a normal capacity to excrete sodium.

IT HAS been fully demonstrated that aldosterone plays an important rôle in sodium retention in cirrhotic patients with ascites. The action of aldosterone takes place mainly at the distal tubular level (1); thus, when proximal acting diuretics are administered, the result is scanty sodium excretion and a large potassium loss (2). Therefore the use of aldosterone antagonists is important, since they permit a larger sodium diuresis with a smaller potassium loss, or none (3, 4). Spironolactone has proved to be the most useful anti-aldosterone therapeutic agent in patients retaining sodium and water (5, 6). In studies of the acute type, progesterone has acted as an aldosterone antagonist (7) and, when administered along with diuretics, has proved useful in the treatment of ascites (8).

The development of new orally administered compounds with progestational activity emphasized the need to carry out this investigation in order to determine the possible anti-aldosterone action of 6-chloro,6-dehydro, 17α-acetoxyprogesterone (RS-1280) (Fig. 1), since it has been reported that this substance is twenty times as potent as progesterone as far as its progestational action is concerned (9).

Received May 12, 1961.

MATERIAL AND METHODS

Five patients with cirrhosis of the liver and ascites were studied in the Metabolic Unit under previously reported conditions (10).

Body weight, urine volume and sodium, potassium, chloride and solute excretion (osmolality) in the urine were measured at 24-hour intervals. Levels of sodium, potassium, chloride, bicarbonate, urea, creatinine, osmolality, hemoglobin and hematocrit were also measured regularly in either serum or blood. Electrocardiograms were obtained at regular intervals.

Body weight was measured under basal conditions on a scale with a maximal error of 100 gm. Sodium and potassium were determined with a lithium internal standard Perkin-Elmer flame photometer; chloride, with the Cotlove chloridometer (11); osmolality, with the Fiske osmometer; serum bicarbonate by the Peters-Van Slyke technique (12); and blood urea, hemoglobin and hematocrit by the usual methods in the clinical laboratory. Urinary nitrogen (Gunning technique) (13), 17-hydroxycorticoids (Silber and Porter

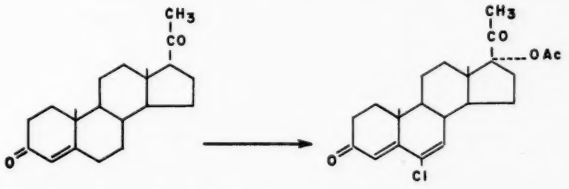


FIG. 1. Chemical configuration of 6-chloro,6-dehydro,17α-acetoxyprogesterone.

technique) (14) and 17-ketosteroids (Holtorff and Koch technique) (15) were also measured.

To assess the functional state of the liver, bromsulfalein retention (16), cholesterol sterification (17) and prothrombin time (18) were periodically measured.

All patients received a constant diet. The 24-hour intake varied from one patient to another, within the following limits: total water (including both drinking water and that derived from the diet), 1430 to 1593 ml.; sodium, 8 to 14 mEq.; and potassium, 61 to 73 mEq.

In every case there was an initial control period during which the capacity of each subject to excrete sodium, water and potassium in the urine was determined, considering the sodium intake and observing the patient's response to thiazide derivatives and mercurial diuretics. Two of the patients were in the stage of sodium normal diuresis (Stage III) and 3 were in the stage of sodium excessive proximal reabsorption (Stage I) (10).

In 3 of the subjects (R.H.R., J.G.A. and A.A.H.), a dosage of 10 mg. per day of RS-1280 was administered for seven to seventeen days; in Subject J.G.A. the dosage was 20 mg. per day for three days and in Subject C.T.F. it was 50 mg. per day for twenty-seven days. Four of the patients, while receiving RS-1280, were given benzhydroflumethiazide simultaneously over a period of three days (10, 20 and 40 mg. respectively) and meralluride (2 ml. intramuscularly on the third day of benzhydroflumethiazide administration). (These diuretics were given during separate three-day periods, once or twice to each subject.) Four patients were given spironolactone to compare the response to this known anti-aldosterone compound with the response to the substance under consideration.[1]

FIG. 2. **a)** First part of the metabolic study in Patient R.H.R. Urine volume, natriuresis (NaO) and kaliuresis (KO) are plotted in heavy lines. The dotted lines represent sodium and potassium intakes. The numbers on the NaO and KO lines indicate the serum values of each ion. On the bottom are plotted body weight changes and the compounds administered; on one level are the dosages of spironolactone (SC-9420), NaCl, water and RS-1280; below is the dosage of benzhydroflumethiazide (10, 20 and 40 mg. daily). The arrow indicates the day on which a 2-ml. dose of meralluride was injected intramuscularly.

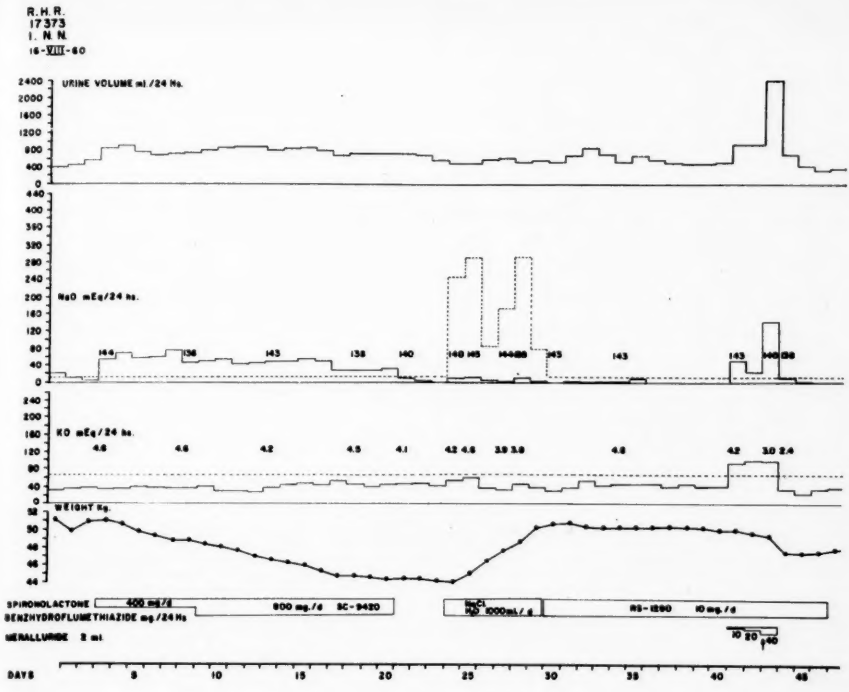
b) Second part of the metabolic study in Patient R.H.R.

RESULTS

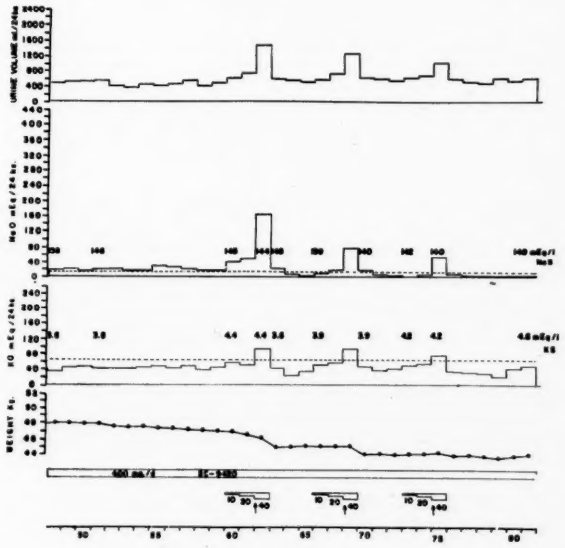
The results are presented in Figures 2 to 5. Patients R.H.R., J.G.A. and C.T.F. were in the stage of sodium excessive proximal reabsorption (Stage I). In Patient R.H.R. (Fig. 2a), increased urine volume (from 570 ml. to 895 ml.) and increased sodium excretion (from 5 mEq. to 77 mEq.) were observed during administration of spironolactone. An excess of sodium was given for a period of six days (total, 1169 mEq.); this caused the body weight to rise gradually to the pre-spironolactone level. RS-1280 was administered during seventeen days in a dosage of 10 mg. per day. There was no increase in sodium diuresis and only a slight weight loss (from 50.5 Kg. to 49.8 Kg.). An increase in sodium diuresis (from 1 mEq. to 142 mEq.) occurred only during the three days when benzhydroflumethiazide, meralluride and RS-1280 were simultaneously administered. Nevertheless, the diuretics also provoked a significant increase in potassium diuresis (from 38 mEq. to 97 mEq.), to the point of producing a negative potassium balance (83 mEq. in three days). On the other hand, when spironolactone was administered again (400 mg. per day for twelve days) (Fig. 2b), increased natriuresis (from 1 mEq. to 27 mEq.) and body weight loss (from 47.9 Kg. to 46.8 Kg.) were observed. These effects were more apparent when the diuretic combined therapy was added to spironolactone; natriuresis increased from 27 mEq. to 161 mEq. and body weight diminished from 46.8 Kg. to 43.5 Kg. During the days when benzhydroflumethiazide and meralluride were given, a negative potassium balance was also observed (27 mEq. in three days), although not as large as the one observed during the administration of RS-1280 along with diuretics. The fact that the "potentiation" combinations gradually became less effective, probably in relation to the decreasing quantity of retained fluid, should be stressed.

In Patient J.G.A., no sodium diuresis occurred when only RS-1280 was

[1] The drugs employed were kindly supplied as follows:
By Syntex, S. A.—6-chloro,6-dehydro,17α-acteoxyprogesterone.
By G. D. Searle and Co.—spironolactone.
By E. R. Squibb and Sons—benzhydroflumethiazide


R.H.R.
17373
I. N. N.
16-VIII-60
URINE VOLUME ml./24 Hs.
NaO mEq/24 hs.
KO mEq/24 hs.
WEIGHT Kg.
SPIRONOLACTONE 400 ml/d
BENZHYDROFLUMETHIAZIDE mg./24 Hs
MERALLURIDE 2 ml.
800 mg./d SC-9420
RS-1280 10 mg./d
DAYS

a



b

given. However, upon administration of RS-1280 in association with benzhydroflumethiazide and meralluride, there was a pronounced increase in sodium diuresis (from 0 mEq. to 118 mEq.), but with a large potassium loss (from 60 mEq. to 116 mEq.). When spironolactone was given (400 mg. per day) during a similar period, a slight increase in natriuresis was observed (from 1 mEq. to 24 mEq.) with no increase in potassium loss (Fig. 3). When spironolactone and diuretics were combined, there was a similar increase in natriuresis (from 18 mEq. to 141 mEq.), also with no increase in potassium loss. When RS-1280, benzhydroflumethiazide and meralluride were given, body weight decreased from 64.6 Kg. to 63.3 Kg. This effect was larger (body weight decreased from 60.4 Kg. to 58.9 Kg.) during a comparable period of combined spironolactone and diuretics therapy. Administration of spironolactone was continued for fifty more days, supplemented by diuretics "potentiation" seven times for short

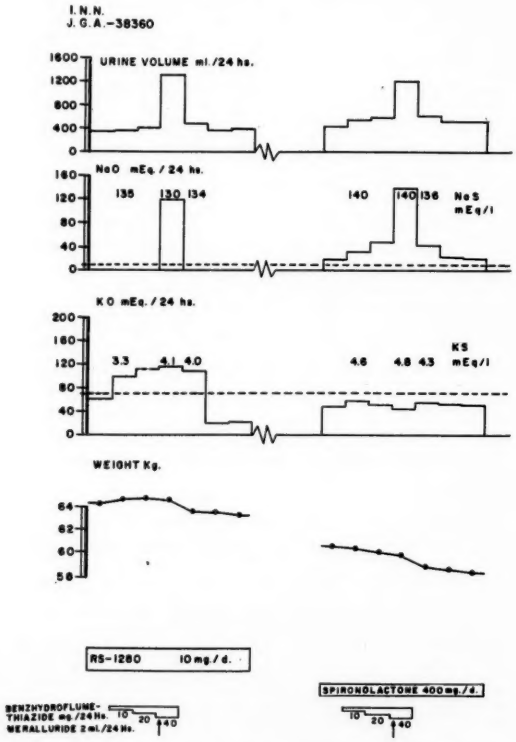


FIG. 3. Two comparable periods during the metabolic study in Patient J.G.A. *Left:* Period of administration of RS-1280 and "potentiated" diuretics. *Right:* Period of administration of spironolactone and "potentiated" diuretics.

periods of three days at three-day or four-day intervals. In those fifty days a striking diminution in body weight was observed (from 58.5 Kg. to 45.8 Kg.) and natriuresis gradually decreased, as in Patient R.H.R.

In Patient C.T.F. (Fig. 4), in spite of the larger dose of RS-1280 (50 mg. per day), no increase in sodium excretion was observed. When benzhydroflumethiazide and meralluride were added, urine volume increased (from 300 ml. to 1030 ml.); there was no increase in sodium excretion and

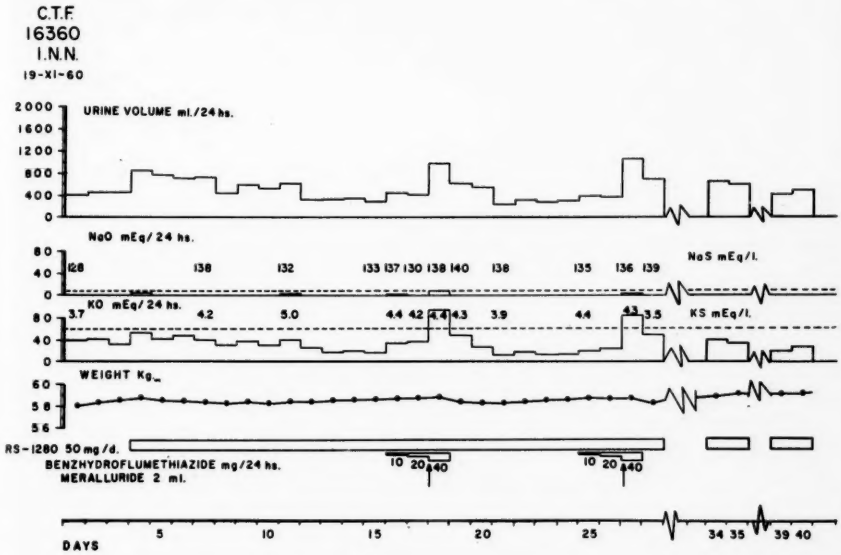


FIG. 4. Prolonged metabolic study in Patient C.T.F. At the extreme right are 2 acute studies. For key, see legend to Figure 2a.

potassium loss was considerable (from 16 mEq. to 93 mEq.). The volume of urine increased from 405 ml. to 868 ml. during the first four days of the 50-mg. dosage of RS-1280, and decreased in the following days; in fact, some reports (8, 19) state that progesterone exerts its maximal effect as an anti-aldosterone agent during the first days of its administration. Accordingly, a 50-mg. dose of the drug was administered daily during two two-day periods, with a three-day interval. During the first of these brief periods, the urine volume increased from 400 ml. to 640 ml. per day, whereas no changes in sodium and potassium excretion were noted. During the second brief period, the urine volume increased from 305 ml. to 495 ml. per day, also with no variations in natriuresis and kaliuresis.

Patients A.A.H. and R.C.S. were in the stage of sodium normal diuresis

(Stage III). In Patient A.A.H. (Fig. 5), during the period of simultaneous administration of benzhydroflumethiazide and meralluride, natriuresis rose from 7 mEq. to 402 mEq. When this patient received 10 mg. of RS-1280 per day for twelve days, the body weight diminished from 45.4 Kg. to 41.6 Kg., the 24-hour volume of urine increased from 640 ml. to 1,438 ml., the 24-hour excretion of sodium increased from 9 mEq. to 120 mEq., and potassim excretion remained unchanged. Upon withdrawal of

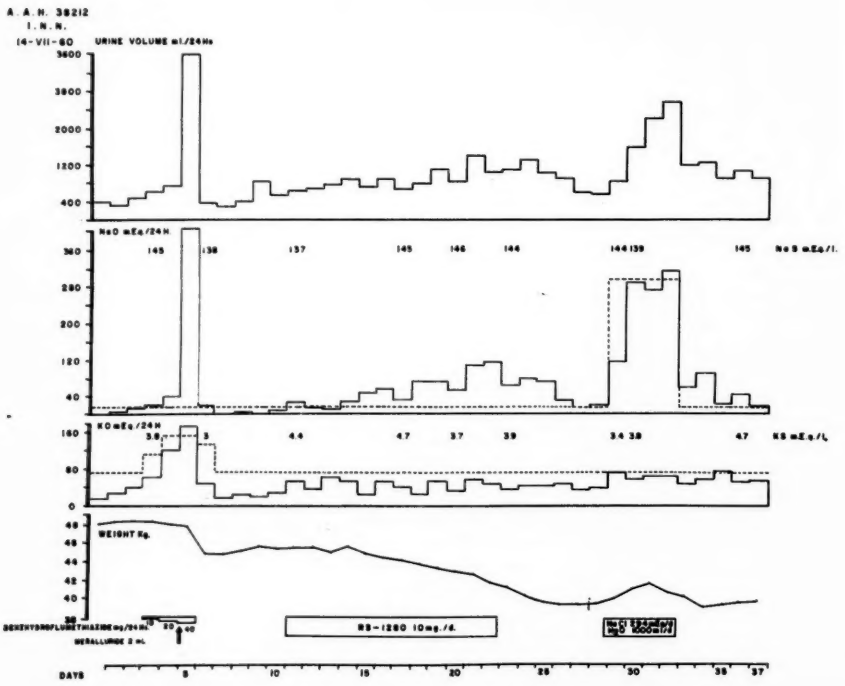


FIG. 5. Metabolic study in Patient A.A.H. For key, see legend to Figure 2a.

RS-1280, natriuresis gradually diminished during the following six days. After this period, sodium chloride (294 mEq. per day in 1,000 ml. of water) was administered intravenously for four days, aiming to raise the body weight to a level similar to the initial one prior to RS-1280 administration, so that spironolactone could be administered in order to compare results obtained with both compounds. The patient failed to gain weight with this procedure and spironolactone was not given. As seen in Figure 5, when Patient A.A.H. received sodium chloride and water in excess, natriuresis increased strikingly, reaching levels of sodium balance. This

increase confirmed that the patient was in the stage of sodium normal diuresis (Stage III). Nevertheless, the increase in urine volume and in natriuresis which occurred during administration of RS-1280 can only be interpreted as a natriuretic effect of the compound; otherwise it would be difficult to explain a 111-mEq. natriuresis while the sodium intake was 14 mEq. per day.

In Patient R.C.S. (Fig. 6), RS-1280 was administered in a dosage of

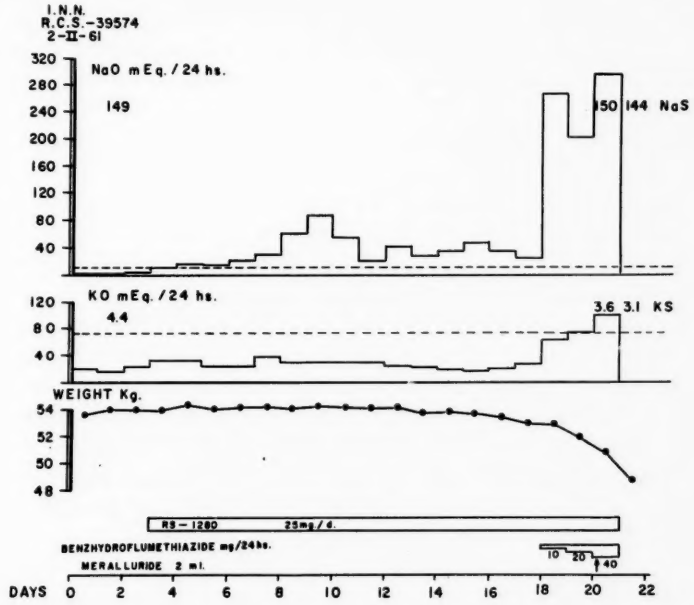


FIG. 6. Metabolic study in Patient R.C.S. For key, see legend to Figure 2a. (Urine volume is not plotted.)

25 mg. daily for eighteen days; there was a gradual increase in sodium excretion which reached 89 mEq. during the seventh day of the administration; from that day on, natriuresis diminished gradually. On the sixteenth day of RS-1280 administration, "potentiated" diuretic therapy was added; increases in urine volume (from 710 ml. to 2,780 ml.) and in sodium diuresis (from 25 mEq. to 295 mEq.) were observed, along with a pronounced potassium loss (from 17 mEq. to 100 mEq.).

The urinary excretion of chloride in every case paralleled the natriuresis. In 3 of the 5 patients (R.H.R., C.T.F. and A.A.H.), during the first four days of RS-1280 administration, urinary osmolality tended to decrease (132 mOsm. per Kg., average).

During or after administration of RS-1280, no changes were observed in the results of liver function tests or in the levels of serum or blood urea, creatinine, bicarbonate, hemoglobin and hematocrit.

In Patients C.T.F., A.A.H. and R.C.S., study of the nitrogen balance showed that it was not negative. In Patients C.T.F., A.A.H. and R.C.S., urinary 17-hydroxycorticoids and 17-ketosteroids were determined daily but no change was observed.

DISCUSSION

Our results show that 6-chloro,6-dehydro,17α-acetoxyprogesterone has no anti-aldosterone action when used alone or in combination with the usual diuretics.

Aldosterone has the following effects: 1) it *decreases sodium diuresis* since it retains sodium in the distal tubule as sodium chloride or exchanges it with potassium or hydrogen (which can be measured in urine as titratable acidity and ammonium); 2) it *decreases urine volume* since water is retained along with sodium; and 3) it *increases potassium and hydrogen excretion* because of the aforementioned exchange. A compound can be called an aldosterone antagonist only if it reverses *all* of the hormonal effects; that is, if it increases natriuresis and urine volume and decreases potassium and hydrogen excretion.

If the compound under consideration reverses only one or a few of the hormonal effects, it cannot be called an aldosterone antagonist. For example, a mercurial diuretic increases sodium and chloride excretion, which represents an "anti-aldosterone effect," but at the same time it provokes a large potassium loss, as aldosterone does. Acetazolamide increases sodium and chloride excretion and decreases titratable acidity and ammonium excretion, thus reversing some of the aldosterone effects; however, like the hormone, it increases the excretion of potassium. Chlorothiazide increases sodium and chloride excretion, also reversing some of the aldosterone effects, but in therapeutic doses it does not modify titratable acidity nor ammonium excretion, and it increases potassium loss. Therefore, none of these diuretics (meralluride, acetazolamide or chlorothiazide) can be regarded as an anti-aldosterone compound.

The alleged anti-aldosterone action of RS-1280 should take place at the renal tubule, since progesterone has been shown to act at that level and to have no influence upon aldosterone synthesis (7).

The best way to detect anti-aldosterone activity in a compound is to give it to adrenalectomized animals or humans, or to patients with Addison's disease receiving aldosterone, or to patients with primary aldosteronism. Nonetheless, in cirrhotic patients with ascites, aldosterone is secreted at

an increased rate (20). These patients lose excessive quantities of potassium when diuretics acting in the proximal tubule are used; therefore, they are good cases in which to study the anti-aldosterone action of a given compound. This action is demonstrated when the excessive loss of potassium is prevented or lowered.

Throughout this study, in the cirrhotic patients who received simultaneously two diuretics acting on the proximal tubule (meralluride and benzhydroflumethiazide), there was a pronounced loss of potassium. When the same patients were given the same diuretics in association with spironolactone, the anti-aldosterone action of the latter compound could be observed, since it usually prevents or diminishes the excessive potassium loss. With these basic criteria in mind, it was found that when RS-1280 is administered in association with diuretics acting on the proximal tubule, the excessive loss of potassium is not prevented or diminished. This indicates the persistence of ionic exchange at the distal tubule. Therefore, it can be stated that RS-1280 is not an anti-aldosterone compound.

Landau *et al.* (21) have pointed out that synthetic progestational compounds lose their progesterone-like anti-aldosterone action when the lateral chain is at carbon 17. This probably explains the inefficacy of the compound studied since it has the alpha-acetoxy radical at carbon 17. Jørgensen and Eilersen (8), in studies of cirrhotic patients treated with mercaptomerin and hydroflumethiazide, observed that progesterone has an anti-aldosterone action. Nevertheless, their results show that while the largest urinary volume and sodium excretion occurred when these compounds were administered along with progesterone, a pronounced potassium loss occurred also—a fact which excludes the anti-aldosterone action of the natural form of progesterone used.

The present study once more points out the importance of knowing the status of the patient in any given case of cirrhosis, with respect to the capacity for water, sodium and potassium excretion. During administration of RS-1280, an increase in natriuresis could be demonstrated in patients with sodium normal diuresis (Stage III) whereas this did not occur in patients with sodium excessive proximal reabsorption (Stage I).

In 3 cases, nitrogen balance was calculated. It was verified that although the patients received from 10 to 50 mg. per day of RS-1280 (equivalent to 200 to 1,000 mg. per day of natural progesterone) for eleven to sixteen days, negative nitrogen balance did not occur—contrary to the results of other authors in studies with progesterone (21). This fact is important because, since 6-chloro,6-dehydro,17α-acetoxyprogesterone does not increase protein catabolism, it has fewer side-effects when used as hormonal therapy (9).

No inhibition of the anterior pituitary occurred during administration of RS-1280 as judged by the unchanged excretions of 17-ketosteroids and 17-hydroxycorticosteroids throughout the study.

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DEMONSTRATION OF 16α-HYDROXYLASE IN THE HUMAN OVARY*†

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ABSTRACT

The presence of 16α-hydroxylase in the human ovary was demonstrated by conversion of 4-C^{14}-progesterone to 16α-hydroxyprogesterone in incubations with tissue minces. Increased 16-hydroxylase activity was found in tissues abundant in follicular and thecal elements. There is suggestive evidence that Stein-Leventhal ovarian tissue contains more enzymatic activity than does normal ovarian tissue.

IN A previous publication (1), we reported the isolation and identification of 17α-hydroxyprogesterone, 20α-hydroxy-4-pregnene-3-one, 17α,20α-dihydroxy-4-pregnene-3-one and androstenedione from incubations of physiologic and pathologic ovarian tissue with progesterone-4-C^{14} as the substrate. Comparison of the morphology and synthetic patterns revealed sufficient increases in androstenedione biosynthesis in the luteinized polycystic ovary to lend support to the concept that C-19 steroids are chiefly responsible for the hirsutism accompanying the Stein-Leventhal syndrome. One major polar compound was not identified at that time. After considerable study it has been characterized as 16α-hydroxyprogesterone. This indicates the presence of an active 16α-hydroxylase in ovarian tissue.

MATERIALS AND METHODS

The conditions of incubation, steroid extraction, purification and derivative formation as well as the criteria for proof of structure have been described previously (1). After representative portions of the ovaries were taken for microscopic examination, the remainder of the ovarian tissue was minced and incubated for four hours in a system modified similarly to that of Hayano *et al.* (1, 2). The substrate was 4-C^{14}-progesterone. Reduced cofactors, generating systems and gonadotropins were purposely avoided. After

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extraction with chloroform and subsequent partition between Skellysolve B:70% ethanol and ether:alkali aqueous systems, preliminary separation of the neutral steroid fraction in the hexane-formamide system of Zaffaroni (3) for twenty-two hours afforded several fractions. The fraction which remained at the origin was rechromatographed in the hexane:benzene-formamide system for twenty-two hours; this procedure separated two major components. The less polar of these was characterized as 17α,20α-dihydroxy-4-pregnene-3-one (1). The more polar compound was designated as O_1 and aliquots of this product obtained from several incubations were pooled to obtain sufficient quantities for subsequent identification and characterization as 16α-hydroxyprogesterone.

IDENTIFICATION

Preliminary studies

Pooled fraction O_1 demonstrated a maximal light absorption at 240 mμ. On chromatography of aliquots of the compound, Rf's were similar to those of authentic 16α-hydroxyprogesterone in the hexane:benzene-formamide (Rf, .07), benzene-formamide (Rf, .29), and benzene:chloroform-formamide (Rf, .45) systems. Acetylation with acetic anhydride and pyridine yielded a substance with the Rf of authentic 16α-hydroxyprogesterone acetate (.13) in the hexane-formamide system. Compound O_1 was unchanged by treatment with periodic acid under conditions shown to cleave vicinal hydroxyl groups (4).

Aliquots of radioactive compound O_1 were chromatographed with small quantities of standard 16α-hydroxyprogesterone, 18-hydroxyprogesterone and 19-hydroxyprogesterone. The radioactivity coincided with that of the 16α-hydroxyprogesterone carrier. Serial portions of the area occupied by the 16α-hydroxyprogesterone carrier were eluted separately, with subsequent determinations of specific activity of each portion as shown in Table 1. This procedure separated most of the radioactive substance from 19-hydroxyprogesterone but not from 18-hydroxyprogesterone.

Dehydration of 16α-hydroxyprogesterone

One drop of glacial acetic acid and 0.5 ml. of concentrated sulphuric acid were added to 165 μg. of standard 16α-hydroxyprogesterone, and the solution left at room temperature for two hours. The acid was then neutralized drop by drop with 10 per cent sodium carbonate. Water was added and the steroid was extracted with chloroform. The acid-treated steroid had a λ max. at 240 mμ (ethanol), an ε max. 1.6 times that of a similar amount of the 16α-hydroxyprogesterone standard, and an Rf similar to that of 16-dehydroprogesterone in the hexane-formamide system. These results are compatible only with the dehydration of 16α-hydroxyprogesterone to form 16-dehydroprogesterone. The increase in ε max. is due to the creation of a double bond conjugated to the 20-carbonyl group. Persistence of λ max. at 240 mμ militates against intro-

duction of a conjugated double bond in the A or B ring. Similar treatment of 18-hydroxyprogesterone, the only possible alternative, resulted in no change in ϵ max., and the acid-treated product did not have the Rf of 16-dehydroprogesterone.

Proof of structure

Proof of structure was undertaken, utilizing the carrier technique of Berliner and Salhanick (5). Authentic 16α-hydroxyprogesterone carrier was added to 25,000 c.p.m. of compound O_1. The specific activities of the mixture and the subsequent derivatives were determined. The techniques for acetylation, oxidation, hydrolysis, and quantitative procedures have been described adequately in the previous publication (1).

Acetylation yielded a compound with the Rf of standard 16α-hydroxyprogesterone acetate in two systems. Subsequent acylase hydrolysis

TABLE 1. SPECIFIC ACTIVITY OF CARRIER 16α-HYDROXYPROGESTERONE

Fraction number*	Steroid (μg.)	Radio activity (c.p.m.)	Specific activity** (c.p.m./μg.)
17	17	148	8.7
18	20	152	7.6
19	7.1	70	8.9

* Fraction number indicates distances in centimeters from the origin of the chromatogram.

** After chromatography with a small aliquot of compound O_1 in the hexane:benzene-formamide system for twenty-two hours.

yielded a compound with the Rf of standard 16α-hydroxyprogesterone in the hexane:benzene-formamide system (twenty-two hours). The carrier area was divided into two portions: *a*) 9–12 cm., and *b*) 13–16 cm. from the origin. The specific activity of each was determined individually. One portion was oxidized with chromic acid, giving rise in poor yield to a steroid with the Rf of standard 16-ketoprogesterone in the benzene-formamide system. The other portion was dehydrated by treatment with concentrated sulphuric acid at room temperature for two hours, as described previously. Chromatography in the hexane-formamide system confirmed the conversion to a substance with an Rf similar to that of 16-dehydroprogesterone. The specific activities of the original carrier mixture and the subsequent derivatives remained constant, as shown in Table 2.

OVARIAN SYNTHESIS

The relative percentages of 16α-hydroxyprogesterone synthesized from progesterone substrate by ovarian tissues of differing morphologic types are shown in Table 3. Appropriate photomicrographs of tissue morphology

TABLE 2. PROOF OF STRUCTURE OF 16α-HYDROXYPROGESTERONE

Reaction	Rf identical with that of	In system	Specific activity* (c.p.m./μM)
—	16α-hydroxyprogesterone	hexane:benzene-formamide (22 hrs.)	4,300
acetylation	16α-hydroxyprogesterone	a) heptane:benzene-formamide b) hexane:benzene-formamide	4,600 4,000
hydrolysis	16α-hydroxyprogesterone	hexane:benzene-formamide (20 hrs.)	a) 4,560 b) 4,420
oxidation	16-ketoprogesterone	benzene-formamide	4,800
dehydration	16-dehydroprogesterone	hexane-formamide	4,980

* After addition of authentic 16α-hydroxyprogesterone to compound O_1 and formation of subsequent derivatives.

have been published previously (1). Specimens 1 and 2 were wedges of normal ovarian tissue taken on the eighth and ninth days of the cycle; they contained numerous small antral follicles without luteinization. Specimen 3 was a wedge of a normal ovary taken on the twenty-sixth day of the cycle (the corpus luteum was in the other ovary). Specimens 4a and 4b were wedges from the uninvolved ovary taken from a 21-year-old woman with amenorrhea and a persistent corpus luteum which had been observed eighteen weeks previously by culdoscopy; they contained numerous small cysts bereft of granulosa and theca cells. Specimen 8 was a wedge from a luteinized polycystic ovary taken from a patient with the clinical findings of the Stein-Leventhal syndrome.[1] Specimen 9 was an

TABLE 3. BIOSYNTHESIS OF 16α-HYDROXYPROGESTERONE AND 17α,20α-DIHYDROXY-4-PREGNENE-3-ONE*

Specimen		Per cent of starting material		
Text No.	Tissue	total origin fraction	16α-hydroxy-progesterone	17α,20α-dihydroxy-4-pregnen-3-one
1	Proliferative ovary (8th day)†	10.0	7.8	2.2
2	Proliferative ovary (9th day)	9.0	7.9	2.1
3	Luteal ovary (26th day)	5.0	5.0	?
4a	Cortex and capsule	5.0	5.0	?
4b	Cortex and medulla (persistent corpus luteum)	4.0	4.0	?
8	Luteinized polycystic ovary	12.0	9.5	2.5
9	Atrophic ovary	0.5	0.3	0.2

* Percentage of labeled progesterone converted to origin fraction, 16α-hydroxyprogesterone and 17α, 20α-dihydroxy-4-pregnene-3-one by ovarian tissues in various physiologic and pathologic states.

† The day indicated in parentheses is the day of the menstrual cycle.

[1] This tissue was supplied by Dr. Michael Leventhal.

atrophic ovary taken from an 81-year-old woman, thirty-two years after the menopause; it contained no follicular structures. Unfortunately, the "origin fractions" of specimens 3, 4a and 4b (containing both 16α-hydroxyprogesterone and 17α,20α-dihydroxy-4-pregnen-3-one) were combined before separation of these two compounds was effected. For this reason only percentages representing the total origin fraction can be expressed for these specimens.

DISCUSSION

The demonstration of active *in vitro* conversion of progesterone to 16α-hydroxyprogesterone indicates the presence of a steroid 16α-hydroxylase in the ovary. In these studies, 16-hydroxylation was associated with the presence of follicular structures, and little conversion was found in specimens bereft of granulosal and thecal elements. Synthesis of 16α-hydroxyprogesterone by the luteinized polycystic ovary (Stein-Leventhal) was approximately 20 per cent greater than that by proliferative phase ovarian tissue, and 100 per cent greater than that by the luteal phase ovary devoid of corpus luteum. Because of the limited number of incubations, it is impossible from these data to conclude that this difference is more than suggestive. Nevertheless, it does appear conclusive that 16α-hydroxyprogesterone is produced by actively synthesizing tissues and is not an artifact.

Substances oxygenated at the 16 position have been isolated previously from natural sources (Table 4). These fall into three categories: phenolic, neutral C-19, and neutral C-21 steroids. The authors are unaware of any natural C-21 substances reported with substitutions at both the 16 and 17 positions, but obviously our survey (Table 4) is incomplete.

The best known 16-oxygenated steroids are found in the phenolic group. Estriol has been isolated from human placenta and pregnancy urine. It is also present in lesser but significant amounts in the urine of nonpregnant women. *In vivo* conversion of administered estrone and 16-ketoestrone to estriol has been reported (8, 9). Slaunwhite and Sandberg demonstrated labeled 16-ketoestrone in the urine of nonpregnant women after administration of 16-C^{14}-estrone during the luteal phase of the menstrual cycle. It is of interest that no conversion was detected in the absence of the corpus luteum (15).

With these considerations, one might reconsider the concept of Hisaw *et al.* (26) on the possible biologic role of estriol. They demonstrated the inhibitory action of estriol on estrone and estradiol and postulated that secretion of estriol in appropriate quantities may serve to prevent excessive estrogenic effects by the more potent estrogens. The demonstration of a 16-hydroxylase in reproductive tissues lends support to this thesis that

will be even more impressive if future studies indicate that this enzyme will also hydroxylate estradiol in the 16 position. In this regard it should be noted that Zander *et al.* (27) isolated both estrone and estradiol from the ovary but failed to demonstrate estriol. Nevertheless, the quantities of tissue extracted were very small.

Rao and Heard (23) reported that swine adrenal homogenates converted progesterone to 16α-hydroxyprogesterone in good yield, and Neher *et al.* extracted 150 mg. of 16α-hydroxyprogesterone from one ton of swine adrenal (21). They also found 5α-pregnane-3β,16α-diol-20-one and its 3α epimer in swine adrenal as well as in the urine of patients with the

TABLE 4. 16-HYDROXYLATED AND RELATED STEROIDS ISOLATED FROM NATURAL SOURCES*

Number	Steroid	Source	Investigator
1	Estriol	Urine, nonpregnant women	Brown *et al.* (6)
	Estriol	Urine, pregnant women	Marrian (7)
	Estriol	Urine, (after admin. of estrone)	Stimmel (8)
	Estriol	Urine, (after admin. of 16-ketoestrone)	Stimmel *et al.* (9)
	Estriol	Human placenta	Collip (10)
2	16α-Hydroxyestrone	Urine, pregnant women	Marrian *et al.* (11)
3	16β-Hydroxyestrone	Urine, pregnant women	Layne and Marrian (12)
	16β-Hydroxyestrone	Urine, (after admin. of estradiol-17β)	Brown *et al.* (13)
4	16-Ketoestradiol-17β	Urine, pregnant women	Layne and Marrian (12)
	16-Ketoestradiol-17β	Human placenta	Diczfalusy and Munstermann (14)
5	16-Ketoestrone	Urine, (after admin. of 16-C^{14} estrone)	Slaunwhite and Sandberg (15)
6	5-Androstene-3β,16:17-triol	Urine, patient with adrenal carcinoma	Hirschmann (16)
7	5-Androstene-3β,16β,17-triol	Urine, normal men	Fotherby (17)
8	3β,16α-Dihydroxy-5-androstene-17-one	Urine, normal men	Fotherby *et al.* (18)
9	3β,7α,16α-Trihydroxy-5-androstene-17-one	Urine, patient with adrenal carcinoma	Okada *et al.* (19)
10	3β,16α-Dihydroxy-5-androstene-7:17-dione	Urine, parient with adrenal carcinoma	Okada *et al.* (19)
11	16-Androstene-3α-ol	Urine, normal men	Brooksbank and Haslewood (20)
12	16α-Hydroxyprogesterone	Swine adrenal	Neher *et al.* (21, 22)
13	16α-Hydroxyprogesterone	Swine adrenal (biosynthesis from C^{14}-progesterone)	Rao and Heard (23)
14	5-Pregnene-3β,16α,20α-triol	Urine, patient with adrenal carcinoma	Hirschmann (24)
15	3β-Hydroxy-16-allopregnene-20-one	Urine, pregnant mare	Klyne and Marrian (25)
16	5α-Pregnane-3α,16α-diol-20-one	a) Swine adrenal	Neher *et al.* (21, 22)
17	5α-Pregnane-3β,16α-diol-20-one	b) Urine, patients with salt-losing adrenogen. syn.	Neher *et al.* (21, 22)

* A cursory survey indicates that many 16-hydroxylated steroids have been reported previously. This table is incomplete.

salt-losing adrenogenital syndrome, and advanced evidence that these compounds have salt-excreting activity (22). The introduction of a 16α-hydroxy group has resulted in a decrease in the salt-retaining activity of synthetic corticosteroids. Furthermore, the steroids with sodium-excreting activity isolated by Neher *et al.* could arise by reduction of the A ring of 16α-hydroxyprogesterone. These findings suggest a possible role for this steroid in salt excretion. Considering the relatively large amount of 16α-hydroxyprogesterone formed in these incubations, it is important that all biologic potencies of the compound be evaluated. Appropriate assays are now in progress in this laboratory.

The finding of most naturally occurring 16-hydroxylated steroids in urine and the demonstration of 16-hydroxylation *in vitro* with liver incuba-

tions (28) have supported the generally held assumption that hydroxylation in this position occurs as a result of hepatic degradation of steroids. In incubations with arrhenoblastoma tissue, using a progesterone substrate, substances demonstrating the light-absorption characteristics of conjugated unsaturation in the A ring and chromatographic polarity similar to that of 16α-hydroxyprogesterone have been isolated (29, 30). Naturally occurring 16-hydroxylated C-19 steroids have been found predominantly in the urine of patients with adrenal carcinoma. The present study demonstrated 16-hydroxylation by ovarian tissue *in vitro* and suggests an increased rate in the luteinized polycystic ovary. These findings indicate that 16-hydroxylation is not exclusively an hepatic function and may, in fact, be associated with hyperactivity of steroid-secreting tissues.

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PERMANENT MYXEDEMA: AN UNUSUAL COMPLICATION OF GRANULOMATOUS THYROIDITIS

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ABSTRACT

A patient who suffered three attacks of acute granulomatous thyroiditis eventually experienced permanent myxedema, as proved by failure of thyroidal uptake of I^{131} to increase after adequate stimulation with TSH. This phenomenon is distinctly unusual, in contrast to the more frequent occurrence of transient myxedema following severe attacks of acute thyroiditis.

IN 1953 Barr (1) stated that in no case of anatomically proven granulomatous thyroiditis had myxedema developed. Other reviews of the subject (2–4) stated that the natural course of the disease is toward spontaneous recovery without demonstrable residual damage to the thyroid gland. More recently, Volpé and associates (5, 6), in discussing the occurrence of transient myxedema following severe granulomatous thyroiditis, emphasized that no proven cases of permanent myxedema had been observed. It is the purpose of this paper to present a case of proven permanent myxedema in a patient suffering three attacks of acute granulomatous thyroiditis.

REPORT OF CASE

A 53-year-old woman was first seen at the Mayo Clinic in May 1959. She stated that in November 1957 she had suffered an illness characterized by painful masses lying on both sides of the larynx, unusual fatigue and malaise, and pronounced nervousness. In December 1957 the erythrocyte sedimentation rate was reported to be 108 mm. in one hour. The painful masses gradually subsided, and in May 1958 the sedimentation rate was reported to be normal. However, chronic malaise continued throughout the year, and in January 1959 she again saw her physician because of the onset of a "puffy face." At this time a serum protein-bound iodine determination was reported to be in the hypothyroid range. In spite of the lack of replacement therapy the facial puffiness gradually decreased, and the malaise subsided prior to her registration at the clinic.

Physical examination revealed that the thyroid gland was diffusely enlarged, quite firm, and nontender. Clinically, the patient appeared to be euthyroid. The sedimentation rate was 21 mm. in one hour (Westergren), the basal metabolic rate (BMR) was −19 per cent, the value for serum protein-bound iodine (PBI) was 5.3 μg. per 100 ml., and that for butanol-extractable iodine (BEI) was 4.5 μg. per 100 ml. A radioiodine (I^{131}) tracer test demonstrated 10 per cent thyroidal uptake in six hours and 18 per cent uptake

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in twenty-four hours. The impression at the conclusion of this first visit was that the patient had suffered an attack of granulomatous thyroiditis followed by transient myxedema, from which spontaneous recovery had occurred; no specific therapy was suggested.

The patient returned in October 1959, stating that two months previously a tender mass had developed in the region of the left lobe of the thyroid gland. The mass had persisted for about two weeks and had been associated with pain radiating to both temporomandibular joints. Her current complaints were of extreme fatigue, husky voice, dry skin, and puffy hands. Examination revealed the thyroid gland to be firm, nontender,

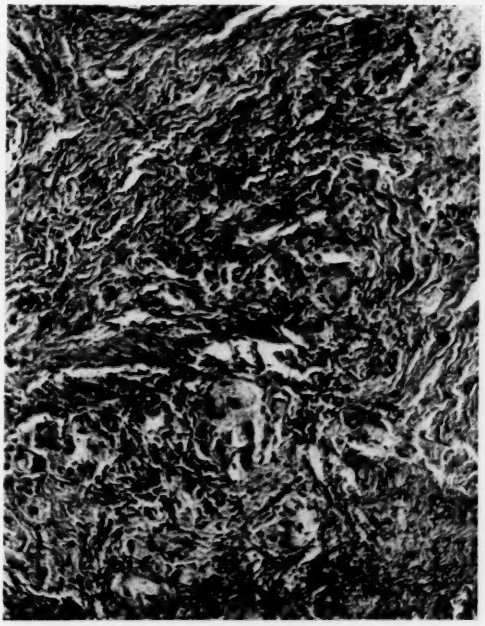

FIG. 1. Thyroid tissue removed by needle biopsy two months after second attack of acute thyroiditis (hematoxylin and eosin; ×160).

and irregular, the right lobe being approximately twice normal size. Clinically, she appeared to have mild myxedema. Laboratory studies at this time revealed the following: the BMR was −16 per cent, the concentration of plasma cholesterol was 281 mg. per 100 ml., the sedimentation rate was 44 mm. in one hour, the PBI level was 4.6 μg. per 100 ml. and the BEI level was 4.2 μg. per 100 ml. of serum. The thyroidal I^{131} uptake was 6 per cent in six hours and 9 per cent in twenty-four hours. Following the subcutaneous injection of 10 units of thyroid-stimulating hormone (TSH), the three-hour uptake was 8 per cent, the six-hour uptake 9 per cent, and the 24-hour uptake 13 per cent. A needle biopsy of the thyroid revealed an inflammatory, destructive process with pronounced fibrosis (Fig. 1). It was expected that the symptoms of mild myxedema would be transitory, so no specific therapy was prescribed.

The patient returned for the third time in May 1960, with complaints of chronic

fatigue, mild intolerance to cold, a husky voice, and the continued presence of dry skin and puffy hands, legs, and face—all suggesting persistence of mild myxedema. The skin was cool and dry, the hair coarse, and the voice husky. Achilles tendon reflexes were slowed. No change was apparent in the thyroid gland since the previous examination. The BMR was −18 per cent, the sedimentation rate 30 mm. in one hour, and the plasma cholesterol concentration 300 mg. per 100 ml. The PBI level was 4.5 μg., and the BEI level 4.1 μg. per 100 ml. of serum. An I^{131} tracer test revealed 7 per cent thyroidal uptake in six hours and 12 per cent in twenty-four hours. Because of continued symptoms of mild myxedema, therapy with desiccated thyroid was started.

Two months later the patient was seen again, stating that she had felt much improved until one week prior to the current visit. At that time she had noted a recurrence of pain in the region of the left lobe of the thyroid gland, with radiation to the left side of the jaw and to the left ear. Examination revealed that the left lobe of the thyroid gland was at least twice normal size; it was firm, smooth, and moderately tender. This finding represented a definite change since the examination two months previously. In addition, tachycardia, elevation of temperature, and pronounced malaise were present. The sedimentation rate was 114 mm. in one hour. The PBI level was 8.4 μg., and the BEI level 6.9 μg. per 100 ml. of serum. Another needle biopsy of the thyroid gland revealed granulomatous thyroiditis with pronounced fibrosis (Fig. 2). An I^{131} tracer test demonstrated 3 per cent thyroidal uptake in six hours and 1.5 per cent in twenty-three hours. To what degree this low thyroidal uptake could be attributed to the ingestion of exogenous thyroid hormone and to what extent to granulomatous thyroiditis was impossible to ascertain.

Therapy for the next six weeks with gradually decreasing doses of prednisone resulted in a prompt decrease of pain and subsidence of fever and malaise. The patient continued to take a full replacement dose of desiccated thyroid.

The patient's last visit to the clinic was in February 1961, at which time she was pleased to report no further recurrences of neck pain. The thyroid gland was of normal size but firmer than usual and of a "granular" consistency; the lobes were of approximately equal size. Clinically, she was euthyroid with the maintenance dose of medication. The sedimentation rate was 26 mm. in one hour, and plasma cholesterol level was 203 mg. per 100 ml. A three-dose TSH test was performed, with the following results:

Date	Dose of TSH (units)	Thyroidal I^{131} uptake (% dose)		
		3 hrs.	6 hrs.	24 hrs.
Feb. 13	—	4	2	1
14	10	3	2	1
15	—	—	—	—
16	10	3	2	1
17	10	2	2	1

The failure of the thyroid gland to respond to potent TSH stimulation was interpreted as indicating permanent and complete loss of thyroidal function. The patient was dismissed with instructions to continue her maintenance dose of desiccated thyroid indefinitely.

COMMENT

The observation that myxedema may follow acute granulomatous thyroiditis is by no means new, but emphasis on the duration of this syndrome has been lacking. McConahey and Keating (7) made reference to 6 of 21 patients with acute thyroiditis who later experienced myxedema,

but no mention was made of its duration or whether TSH-stimulation studies had been carried out. Lindsay and Dailey (8) observed myxedema in 10 of 13 patients with granulomatous thyroiditis who had been treated surgically, and 3 of 12 patients treated medically required permanent replacement therapy for myxedema; however, TSH studies were apparently not performed. Woolner and associates (9) reported the development of

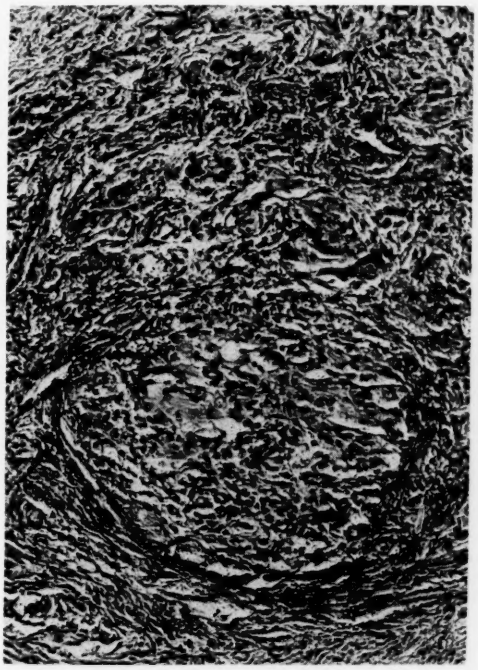

FIG. 2. Tissue obtained at time of second needle biopsy of thyroid, during course of third attack of acute granulomatous thyroiditis (hematoxylin and eosin; ×160).

myxedema in 12 of 73 patients; 2 of the 12 had unilateral thyroid resections, 9 had bilateral subtotal resections, and 1 had only a needle biopsy (whether this last patient had permanent myxedema was not stated). Bergen (10) recently reviewed the pertinent literature and stated that permanent myxedema following acute granulomatous thyroiditis had been reported only twice—once in a patient who received radiation therapy and once in a patient who had been treated with thiouracil. Bergen stated that mild myxedema may occur during the recovery phase, especially in patients who have received radiation therapy, but that these patients almost invariably have only temporarily diminished thyroidal function.

The temporary nature of the myxedema was emphasized by Volpé

and associates (5, 6), who observed myxedema in 11 of 56 patients with granulomatous thyroiditis. Seven of these 11 were not treated during the myxedematous stage, and a euthyroid state was gradually regained over a period of two to seven months. Four of the 11 were treated with desiccated thyroid; 2 of the 4 discontinued its use successfully after one year, and the other 2 refused to undergo a trial period without medication. The reports cited would certainly indicate that permanent myxedema following acute granulomatous thyroiditis is most unusual in patients who have not received surgical therapy. On the other hand, transient myxedema following severe acute granulomatous thyroiditis is apparently not rare.

Although more than one attack of acute granulomatous thyroiditis is not uncommon, the present case is distinctly unusual in that the patient suffered three separate, acute episodes of this disorder. Transient myxedema is most likely to follow clinically severe attacks of granulomatous thyroiditis. The successive injuries of the repeated attacks were probably of great importance in destroying the thyroid parenchyma and producing permanent myxedema in this case.

Several studies of the effect of TSH in acute thyroiditis have been reported, with the observation that a significant response to TSH stimulation during the active phase of acute thyroiditis is unusual (11–13). This is not surprising, for any patient with a primary form of myxedema would be expected to be under at least physiologic TSH stimulation; an increment in available TSH would not necessarily be expected to cause increased trapping of I^{131} by the damaged thyroid parenchyma. TSH stimulation after the active phase of the disease has subsided would be expected to stimulate the remaining or regenerating thyroid parenchyma, even in the presence of exogenous thyroid hormone (14).

Hashimoto's thyroiditis can occasionally present as a painful thyroidal swelling associated with an elevated erythrocyte sedimentation rate, thereby masquerading as granulomatous thyroiditis (15). This possibility was excluded in the present case by study of tissue specimens. The first needle biopsy (Fig. 1) revealed an inflammatory destructive process with fibrosis which was consistent with, though not diagnostic of, granulomatous thyroiditis. It is well known that the activity of the disease and the pathologic features may vary considerably in different areas of the gland (9, 16); had more tissue been available for examination, a diagnostic picture might have been seen. The findings of the second biopsy (Fig. 2) were entirely compatible with the clinical diagnosis. Tubercle formation, such as was noted in this biopsy specimen, has not been observed in the "fibrous" variant of Hashimoto's thyroiditis, in which epithelial destruction is a prominent microscopic feature.

It is worth while to emphasize that should myxedema occur following

an attack of acute thyroiditis, normal function of the thyroid gland can ordinarily be expected to resume spontaneously in two to seven months (5, 6). If symptoms of myxedema are severe enough to warrant treatment, this should be undertaken only on a temporary basis. Seven or more months after the acute attack of thyroiditis, therapy should be withdrawn or TSH studies should be conducted to determine whether the thyroid gland has once again regained the ability to produce hormones normally.

Acknowledgment

I am indebted to Dr. L. B. Woolner for his interpretation of the pathologic material.

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ABNORMAL IODOPROTEINS IN THE BLOOD OF EUMETABOLIC GOITROUS ADULTS*

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ABSTRACT

The serum of 5 eumetabolic goitrous adults was found to contain appreciable quantities of butanol-insoluble iodine. The thyroidal uptake of I^{131} was increased in these patients, but was suppressed by the administration of triiodothyronine. Three subjects exhibited concentrations of serum PBI^{127} and 72-hour PBI^{131} which were in the thyrotoxic range. Following administration of I^{131}, from 26 to 60 per cent of serum radioactivity migrated in the albumin area during zonal electrophoresis of serum. This material was detectable as long as twenty-eight days after administration of the tracer dose. In the serum of normal subjects there was also a radioiodinated compound which migrated with albumin, but which was butanol-soluble and disappeared from the circulation within seven days after administration of I^{131}. The abnormal moiety in the serum of the goitrous subjects was subjected to butanol extraction, hydrolysis, electrophoresis in the presence of Cohn fraction IV-6, chromatography in butanol:water, salting-out by phosphate buffer, and precipitation with anti-human-albumin horse serum. The material appears to be an iodinated peptide or protein of thyroidal origin with several characteristics of an albumin.

ALTHOUGH goitrous hypothyroidism has been known for many centuries (1), insight has been gained only recently into the defects which may underlie the disorder. Five abnormalities have been defined. Four of them directly affect the intrathyroidal metabolism of iodine (2–5),

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but the fifth appears to reside in the synthesis or proteolysis of thryroproteins (6). Affected subjects produce and release abnormal peptides or proteins into the circulation which, unlike the iodine compounds normally present, are poorly extractable by acidic butanol. Most persons in whom this defect has been discovered have been goitrous cretins with mild to severe hypometabolism (7).

It has been suggested that related but milder defects in hormonogenesis might underlie a variety of goiters found in eumetabolic adults. This postulate is supported by the consanguinity of all variants of goitrous cretinism and by the finding of eumetabolic goitrous adults in affected families who exhibit qualitatively similar aberrations in the synthesis of thyroid hormone (5).

Among goitrous adults who were surveyed for an inborn error of hormonal synthesis, some eumetabolic subjects were found who revealed either paradoxically high concentrations of serum protein-bound iodine (PBI) or unusually rapid rates of thyroidal accumulation or release of I^{131}. The incidence of this complex of findings was rare, since only 12 persons among several hundred goitrous adults exhibited it. Of these, 5 were found to have abnormal circulating iodinated materials in clearly detectable amounts.

METHODS

The patients studied were 4 women and 1 man with the foregoing characteristics. The 24-hour thyroidal accumulation of I^{131} was measured before and during administration of triiodothyronine (T_3). A daily dose of 75 μg. of T_3 was given orally for from seven to ten days. Concentrations of serum PBI and of butanol-extractable I^{127} (BEI) were determined before and during suppression of the pituitary-thyroid system with T_3. Concentrations of serum PBI^{131} were determined by precipitation with trichloracetic acid seventy-two hours after administration of the first tracer dose. For characterization of circulating radioactivity, sera were obtained at intervals after administration of 100 to 500 μc. of I^{131} and subjected to 1) extraction with acidic butanol (brought to pH 1.0 with 10 per cent H_2SO_4) containing carrier thyroxine (T_4); 2) electrophoresis in powdered cellulose columns (8) or in replicate filter-paper strips employing either barbital or tris-hydroxymethylaminomethane-maleate (TRIS-maleate) buffers (pH 8.6, ionic strength 0.10); 3) ammonium sulfate fractionation; and 4) extraction with acidic butanol of fractions obtained by electrophoresis or by salting out. Butanol extracts were evaporated to dryness *in vacuo* and redissolved in ammoniacal methanol (2*N* NH_4OH:methanol; 1:7, v./v.). The volume of methanol was reduced by evaporation in a stream of nitrogen and the residual was chromatographed on Whatman #1 filter-paper in an ascending solvent system composed of either butanol:acetic acid:water (78:10:12, v./v.) or butanol:dioxane:ammonia (80:20, v./v. saturated with 2*N* NH_4OH) (9). Iodine-containing fractions obtained by zonal electrophoresis or by salting-out were also directly chromatographed in butanol:water (52:48, v./v.) on Whatman #3 filter-paper (10). Aliquots of these fractions were also subjected to tryptic hydrolysis[1] at 37° C. for twenty-four to forty-eight hours.

[1] Bovine pancreatic trypsin (Tryptar, Armour), 30 mg. per ml. of barbital buffer, pH 8.6, ionic strength 0.1.

The digests were extracted with acidic butanol, concentrated and chromatographed as described. Comparable control studies were made of sera obtained from eumetabolic nongoitrous adults. In view of the earlier reported finding of I^{131} localized in the albumin zone during electrophoresis of sera obtained from normal subjects shortly after the administration of I^{131} (11), sera from 4 control subjects were obtained one, two and/or three days or more after administration of I^{131} and subjected to column electrophoresis. The distribution of radioactivity among fractions collected from the column was then assessed.

Organic I^{131} in the sera of Subject 1 was precipitated with various concentrations of neutral phosphate buffer (12). The capacity of Cohn fraction IV-6 (Method 10) (13), a plasma fraction rich in thyroxine-binding globulin (TBG) (14), to compete with the abnormal iodinated moiety for organic I^{131} was evaluated as follows. A 20-mg. aliquot of lyophilized Cohn fraction IV-6 was dissolved in a 1.0-ml. sample of serum. The IV-6 supplemented serum was subjected to zonal electrophoresis and the distribution of endogenous radioactivity was quantified by counting pooled 1.0-cm. segments obtained from replicate paper electrophoretograms. Concurrently, 1.0 ml. of the unsupplemented serum was subjected to electrophoresis and the distribution of radioactivity was assessed. For comparison, the serum from a normal subject was enriched with I^{131}-labeled T_4 to a concentration of 27.5 μg. per 100 ml., in order to produce a similar distribution of serum radioactivity during zonal electrophoresis. To a second 1.0-ml. sample of serum enriched with labeled T_4 was added 20 mg. of fraction IV-6. The sample was subjected to zonal electrophoresis followed by assessment of the distribution of radioactivity.

Albumin in the serum of Subject 1 was precipitated with optimal quantities of a potent anti-human-albumin horse serum[2] (15). After collection of the precipitate by centrifugation (18,000 $\times$ g), the percentage of total serum radioactivity recovered in the precipitate was measured. The precipitate was then subjected to exhaustive extraction with acidic butanol and the remaining radioactivity was measured.

RESULTS

Results are summarized in Tables 1 and 2. Additional characterizations of the abnormal iodinated materials are depicted in Figures 1–4. Four of the 5 subjects had diffusely enlarged, firm thyroid glands. Subject 4 had a distinctly multinodular gland. No bruit was heard over any gland. The 24-hour thyroidal uptake of I^{131} ranged from 50 to 70 per cent and was within the range characteristic of thyrotoxicosis in 2 cases (Subjects 1 and 5). Serum concentrations of PBI ranged from 6.5 to 12.8 μg. per 100 ml. In 3 of the 5 subjects the values were 10 μg. per 100 ml. or greater (Subjects 1, 2 and 5). Despite these laboratory findings, no patient showed clear-cut evidence of Graves' disease or thyrotoxicosis although Subject 2 had earlier received an 18-month trial of antithyroid therapy with propyl-

[2] The antiserum produced a single band with human serum during agar gel diffusion in the Ouchterlony plate. At equivalence, 0.75 ml. of antiserum precipitated all the protein from 1.0 ml. of a 20 mg. per ml. solution of ARC human serum albumin. Obtained through the courtesy of the Massachusetts State Department of Health, Protein Foundation, Inc.

TABLE 1. SEVERAL PARAMETERS OF IODINE METABOLISM IN GOITROUS EUMETABOLIC ADULTS WITH BUTANOL-INSOLUBLE SERUM IODINE

Subjects			Control values					Values during T_3 suppression			
Case	Age & sex	BMR (%)	Thyroidal 24-hr. I^{131} uptake (% dose)	Serum PBI (μg./100 ml.)	Serum BEI (μg./100 ml.)	PBI minus BEI (μg./100 ml.)	Serum 72-hr. PBI^{131} (% dose/L)	Thyroidal 24-hr. I^{131} uptake (% dose)	Serum PBI (μg./100 ml.)	Serum BEI (μg./100 ml.)	PBI minus BEI (μg./100 ml.)
Goitrous Eumetabolic Adults											
1	58 F	−16	60	10.2	7.1	3.1	0.96	4	8.8	4.0	4.8
2	32 M	+ 4	50	12.8	8.1	4.7	0.37	8	9.1	5.9	3.2
3	34 F	−10	51	7.7	5.0	2.7	0.45	3	6.4	4.5	1.9
4	37 F	− 6	51	6.5	5.1	1.4	0.30	10	6.0	4.7	1.3
5	38 F	+10	70	10.6	6.0	4.6	0.88	14	7.6	4.0	3.6
Nongoitrous Eumetabolic Controls											
A	68 M	—	20	5.0	4.8	0.2	—	—	—	—	—
B	54 F	—	32	6.2	5.5	0.7	—	—	—	—	—
C	52 F	—	22	6.0	5.7	0.3	—	—	—	—	—
D	48 F	—	35	7.2	6.0	1.2	—	—	—	—	—

thiouracil. Basal oxygen consumption in these subjects ranged from −16 to +10 per cent.

After administration of T_3 for at least seven days, the 24-hour thyroidal uptake of I^{131} was suppressed to less than 14 per cent of the administered dose. At the same time the serum concentrations of both BEI and PBI

TABLE 2. SERUM ORGANIC I^{131} LEVELS IN GOITROUS EUMETABOLIC ADULTS WITH BUTANOL-INSOLUBLE SERUM IODINE

	Percentage of serum organic I^{131}													
	Days after tracer dose													
	1	2	3	4	5	6	7	8	9	10	11	12	23	28
Goitrous Eumetabolic Adults														
1. % butanol-soluble	69		73		73		74			74		67	59	69
1. % in albumin zone*	35		—		40		39			45		—	42	44
2. % butanol-soluble	85		80		76				85					
2. % in albumin zone	55		60		48				40					
3. % butanol-soluble	65		[illegible]		75				76					
3. % in albumin zone	36		[illegible]		40				26					
4. % butanol-soluble	79		85				82					85		
4. % in albumin zone	52		45				42					35		
5. % butanol-soluble			70			65					65			
5. % in albumin zone			60			47					52			
Nongoitrous Eumetabolic Controls														
A. % butanol-soluble	90	90	97			94								
A. % in albumin zone	24	15	<10			<10								
B. % butanol-soluble	87	—	92				86							
B. % in albumin zone	30	25	20				<10							
C. % butanol-soluble	88		96				84							
C. % in albumin zone	34		22				<10							
D. % butanol-soluble	93	92	96			96								
D. % in albumin zone	25	27	12			<10								

* During zonal electrophoresis in filter-paper or in cellulose column, TRIS-maleate or barbital buffer, pH 8.6, ionic strength, 0.10.

tended to decrease. The amount of butanol-insoluble I^{127} (PBI minus BEI) decreased somewhat in Subjects 2, 3 and 5, but increased in Subject 1.

Concentrations of PBI^{131} at seventy-two hours were greatly increased, ranging from 0.30 to 0.96 per cent of the administered dose per liter of serum. Butanol solubility of I^{131} in sera obtained from the goitrous subjects over periods ranging from nine to twenty-eight days after administration of I^{131} was generally subnormal. Values for sera obtained on the last day of the study ranged from 65 to 85 per cent of total serum radioactivity. Comparable estimations of butanol solubility of I^{131} in the sera of control subjects yielded values which ranged from 84 to 97 per cent of total serum radioactivity.

Not only were appreciable quantities of albumin-migrating I^{131} observed in the sera of the goitrous subjects but a similar moiety was found in the sera of control subjects. However, several differences were noted in the behavior of the compound in the two cases. Whereas in the former group it represented from 35 to 55 per cent of serum radioactivity twenty-four hours after administration of the tracer, it accounted for from 24 to 34 per cent in the control group. Although it was detectable in the sera of goitrous subjects for the entire period of study of from day 9 to day 28 it decreased to less than 10 per cent of protein-bound radioactivity in normal subjects by the seventh day. Moreover, despite the presence of this compound early after administration of I^{131}, the butanol solubility of serum radioactivity in the control subjects was normal.

In Subject 1, 42 per cent of the serum-I^{131} was precipitable by anti-human-albumin horse serum. Sixty per cent of the precipitated radioactivity was insoluble in acidic butanol. Appreciable quantities of I^{131} remained at the origin when sera of Subjects 1, 2 and 5 were chromatographed in the butanol:water system. Control sera obtained seventy-two hours after administration of I^{131} revealed no immobile component (Fig. 1). Low levels of serum radioactivity precluded similar studies of sera from the remaining goitrous subjects.

Addition of TBG-rich Cohn fraction IV-6 failed to alter the distribution of radioactivity among the protein fractions during electrophoresis of the endogenously labeled serum of Subject 1. In contrast, Cohn fraction IV-6 readily abstracted albumin-bound I^{131}-labeled T_4 in normal serum (Fig. 2).

When compared with the concentration required for precipitation of organic I^{131} in the serum of a normal subject, neutral phosphate precipitation of the serum of Subject 1 revealed a labeled component which required an abnormally high concentration of the buffer for precipitation (Fig. 3). Endogenously labeled compounds extractable with butanol from either TBG or albumin, prepared by column electrophoresis, migrated like I^- and

T_4. Some radioactivity was also noted in the monoiodotyrosine (MIT) and diiodotyrosine (DIT) areas. Compounds extractable from the prealbumin zone isolated by electrophoresis in TRIS-maleate buffer migrated like I^- and T_4. After tryptic hydrolysis of the albumin fraction of Subject 4, iodinated materials in both the MIT and DIT zones were identified (Fig. 4). Low, possibly insignificant, counts were obtained in the T_4 and T_3 areas.

DISCUSSION

Several unusual findings distinguished the present patients from the clinic population of nontoxic goitrous adults. Thyrotoxicosis was suspected

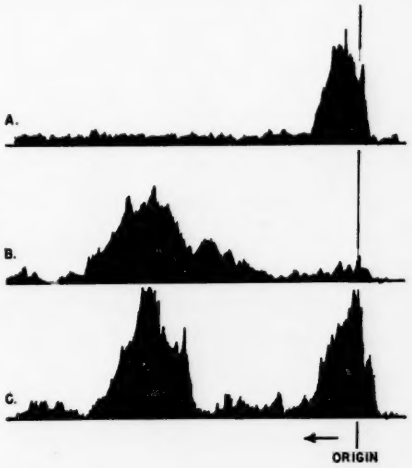

FIG. 1. Chromatography (butanol: water) of the serum of a goitrous eumetabolic adult with butanol-insoluble I^{131}. **A)** endogenously labeled human thyroglobulin. **B)** endogenously labeled normal serum. **C)** serum of subject with butanol-insoluble I^{131}. Both sera were obtained seventy-two hours after administration of 500 μc. of I^{131}.

in several because they displayed high levels of serum PBI, high 24-hour thyroidal uptakes of I^{131}, or both. Indeed, 1 patient had received a therapeutic trial with propylthiouracil. However, signs of Graves' disease were absent and basal oxygen consumption was normal in all cases. That the syndrome was not a nontoxic variant of Graves' disease was suggested by the suppression of the thyroidal accumulation of I^{131} by triiodothyronine. Examination of these patients' sera revealed high concentrations of PBI^{131} and subnormal solubility of PBI^{127} and PBI^{131} in acid butanol. Moreover, appreciable amounts of albumin-bound I^{131} were found during zonal electrophoresis of the sera, and this material was demonstrable for long periods after administration of a large tracer dose of I^{131}. Since the patients were eumetabolic, it was apparent that the abnormal iodinated material was, at most, only weakly calorigenic.

Although the role played by the iodinated compound in the dynamics of iodine metabolism cannot be defined quantitatively from the present

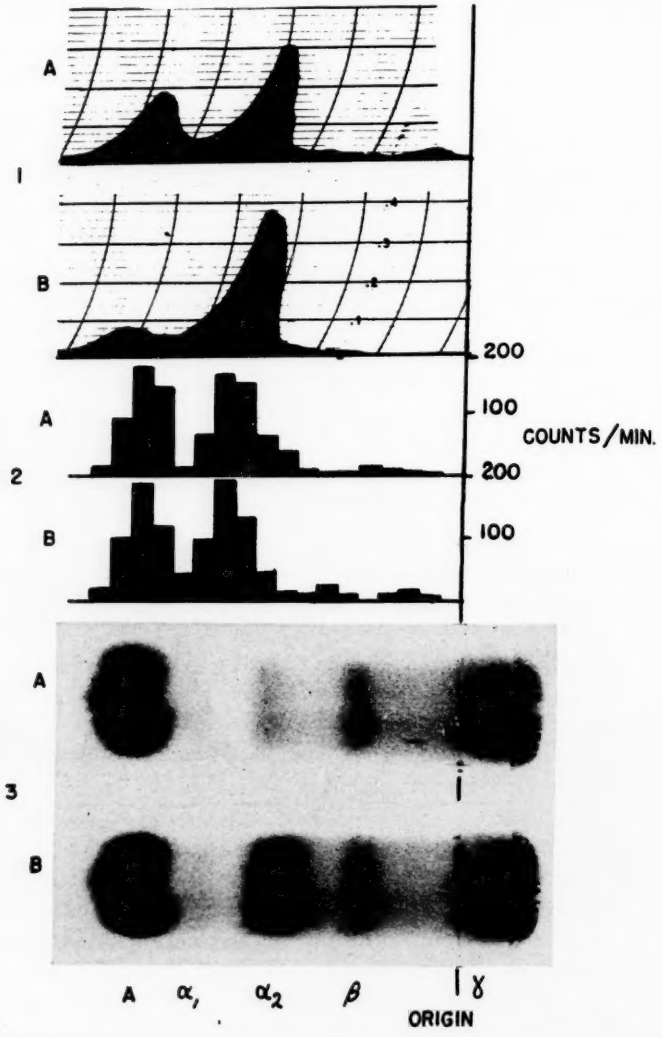

FIG. 2. Effect of added TBG-rich Cohn fraction IV-6 on the electrophoretic distribution of serum organic I^{131}. **1)** (top) Scans of radioactivity in normal serum enriched with tracer and stable T_4 to a concentration of added T_4 of 27.5 μg. per 100 ml.: A—unenriched serum; B—fraction IV-6 enriched serum. **2)** (middle) Counts of radioactivity in endogenously labeled serum of subject with butanol-insoluble I^{131}: A—unenriched serum; B—fraction IV-6 enriched serum. Note displacement of radioactivity from albumin to TBG in normal serum by addition of fraction IV-6, and failure of displacement in serum of subject with the butanol-insoluble I^{131}. **3)** (bottom) Bromphenol blue stained electrophoretograms: A—normal serum; B—fraction IV-6 enriched serum (barbital buffer, pH 8.6).

studies, the development of goiter and laboratory evidences of hyperfunction of the thyroid gland appear to be due to the production and release of the abnormal material into the circulation. That the compound was produced by the thyroid was suggested although not established by: 1) its appearance in the circulation in a high concentration and at a correspondingly high specific activity after a tracer dose of I^{131} (16); 2) its content, after tryptic hydrolysis, of MIT and DIT in nearly equal amounts; and 3) the decrease in PBI-BEI difference during T_3 suppression which occurred in 3 of the 5 subjects. If thyroid function were directed partly toward synthesis of this substance, activation of the pituitary-thyroid system would lead ultimately to compensatory goiter and hyperfunction. In contrast to

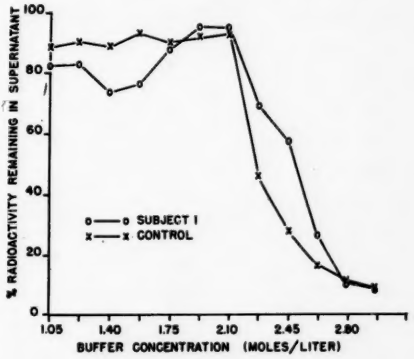

FIG. 3. Neutral phosphate precipitation of endogenous organic I^{131} in the serum of a goitrous eumetabolic adult with butanol-insoluble I^{131}, compared with that in the serum of a normal adult.

sporadic goitrous cretins (3–6, 17–20), the present patients achieved production of normal quantities of hormone.

Several additional observations resulted from further characterization of the abnormal compound and from the control studies performed in normal persons. During studies of the 4 normal subjects, an albumin-migrating I^{131} moiety was also found which was similar to that described by others in the circulation of some normal or thyrotoxic subjects after the administration of I^{131} (11, 21). This material differed from that of the goitrous patients and from that described in certain instances of thyroid cancer (22) and in goitrous cretins with butanol insoluble serum-iodine (6, 7, 17), in that it was soluble in butanol and disappeared from detection in about one week. It appears, therefore, that electrophoretic demonstration of such albumin-migrating materials early after administration of a tracer dose of I^{131} cannot be construed as distinctly abnormal.

It was hoped that simultaneous determination of concentrations of serum PBI and BEI would aid in screening goitrous patients for the abnormality. However, the percentage of serum radioactivity associated with albumin during electrophoresis usually exceeded the percentage of butanol-

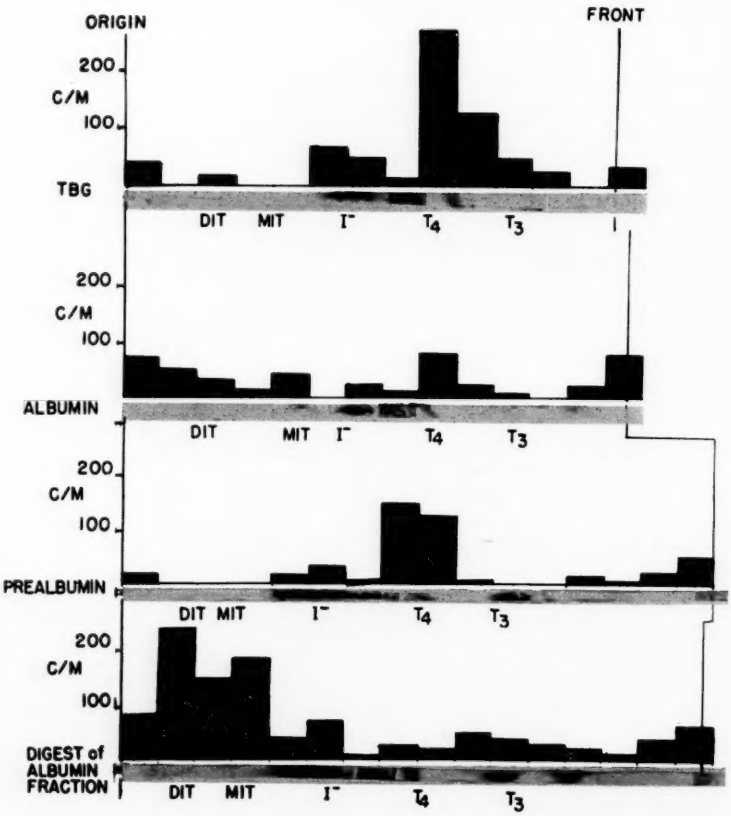

FIG. 4. Chromatography of serum iodine in butanol:dioxane:ammonia. Electrophoretic fractions of 10 ml. of serum obtained from Subject 5 nine days after administration of I^{131} were extracted with acidic butanol, concentrated and chromatographed. The albumin residue after extraction was submitted to alkaline hydrolysis in trypsin for forty-eight hours, then prepared as described, and chromatographed.

insoluble material found during either I^{127} or I^{131} analyses. Moreover, in at least 1, and possibly 2 instances (Subjects 3 and 4), the PBI-BEI difference was not greater than that found in normal sera; and in Subjects 2 and 4, the butanol solubility of serum I^{131} reached the range found in normal subjects. Thus, the difference between concentrations of PBI and BEI and the solubility of serum organic I^{131} in acid butanol appear to be uncertain indicators of less than large amounts of the abnormal compound.

Since the various further characterizations of the abnormal material were performed at different times and in different subjects, the behavior of the compound in the serum of one patient may have differed from that of an

electrophoretically similar compound in the serum of another. Within this limitation it appears that the moiety is an iodinated peptide or protein of thyroidal origin or an iodinated moiety in an unusual, tight association with albumin. That radioactivity localized in the albumin zone during electrophoresis was not contained in albumin-bound thyronines was indicated by its low solubility in acidic butanol. That it was not albumin-bound thyroxine was suggested by the failure of added Cohn fraction IV-6 to abstract radioactivity from albumin to the area of the thyroxine-binding globulin. The protein-like nature of the material was inferred from its immobility during chromatography in the butanol:water system (10). The electrophoretic mobility of the compound excluded its identity with thyroglobulin. Further, the abnormal curve of neutral phosphate-precipitable radioactivity indicated that it could be an iodo-albumin (12). Immunologic study of the serum of Subject 1 did not refute this possibility.

It was not possible to correlate the chemical findings with thyroidal histologic findings or with the chemical composition of thyroproteins. A possible hereditary background of the disorder also could not be examined. Consequently, it is uncertain whether the defect might be congenital or acquired. It is conceivable that during the recovery phase of subacute thyroiditis such a combination of laboratory events could occur. Unfortunately, knowledge of the character of abnormal circulating organic iodine which may appear in this disease is incomplete (23, 24). Similarly, the composition of serum iodine in patients with chronic thyroiditis has not been studied in detail, although release into the circulation of abnormal iodoproteins occurs in this disease (25). However, none of the present group had at any time signs or symptoms of subacute thyroiditis or laboratory evidences of Hashimoto's disease.

Recently, DeGroot and Stanbury (6) and DeGroot and Carvalho (25) have reviewed the recorded cases of goitrous cretinism with butanol-insoluble serum iodine. In the thyroid glands of their own cases they found a noniodinated or slightly iodinated albumin-like compound. They also found the compound in certain colloid goiters and follicular adenomas. Moreover, the sera of certain other patients with colloid goiters contained up to 20 per cent of butanol-insoluble I^{131}. Although the precise relationship between the intrathyroidal albumin-like compound and the butanol-insoluble serum iodine has not been established, there is strong inference that they are identical. Robbins, Wolff and Rall in earlier studies demonstrated nonthyroglobulin iodoproteins in transplantable thyroid tumors of rats and in sheep and human thyroid glands (27, 28). They noted that the compound from human subjects resembled that found in the serum of certain patients with thyroid carcinoma (10) and in a patient with congenital goiter (6). Evidence that the intrathyroidal and circulating com-

pounds were the same was suggested to these workers by the finding that the rat thyroprotein, although different from that of other species, resembled the iodoprotein found in the blood of rats with thyroid tumors.

From the foregoing studies, it is clear that there are several disorders of the thyroid gland in both animals and man in which there is increased thyroidal content of an albumin-like material and, occasionally, the presence in the serum of an albumin-like iodinated moiety. When, as in the present cases, the abnormal material is present in large amounts, there occurs an increase in both the thyroidal uptake of I^{131} and the concentration of serum PBI. The defect, although unusual, is thus another example in the growing list of factors which can pathologically alter the PBI level in eumetabolic subjects. More importantly, these findings support the view that the synthesis and release of abnormal thyroproteins may play an important role in the genesis of certain nontoxic goiters in adults (6, 7, 26–28).

Acknowledgments

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STUDIES OF THYROID AND SYMPATHETIC NERVOUS SYSTEM INTERRELATIONSHIPS. I. THE BLEPHAROPTOSIS OF MYXEDEMA*

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ABSTRACT

Blepharoptosis (drooping of the upper eyelid) was observed in 20 of 27 patients (74 per cent) with untreated myxedema of various types and in 6 of 18 myxedematous patients (33 per cent) treated with desiccated thyroid. The blepharoptosis was characteristically of modest degree and could be overlooked easily. Lid edema, although frequent, was not always associated with ptosis. The tarsal fold was intact and normal voluntary and conjugate lid elevation was possible. Phenylephrine hydrochloride, when instilled into the conjunctival sac, corrected the ptosis in all cases. Electromyograms of the levator palpebrae superioris muscles were normal in 4 patients. Urinary catecholamine excretion was normal in 4 patients. Intravenous infusion of epinephrine did not correct the ptosis in 6 patients. The evidence suggests that blepharoptosis in myxedema results primarily from diminished tone of the sympathetic nerve fibers to Müller's superior palpebral muscle rather than from an abnormality of the levator palpebrae superioris muscle. Blepharoptosis appears to be the converse of the lid retraction of hyperthyroidism, and is a valuable clinical sign of myxedema. It is further evidence of the relationship between the thyroid hormone and the catecholamines of the sympathetic nervous system.

THE paucity of eyelid signs in myxedema is in sharp contrast to the multiplicity of such signs in hyperthyroidism. The best known lid sign in myxedema is edema (2). Narrowing of the palpebral fissure has also been described and has been attributed either to edema or to thickening of the upper eyelid (3–5). In our experience, narrowing of the palpebral fissure is found quite frequently in myxedema, is of diagnostic value, and is the result of blepharoptosis, *i.e.*, drooping of the upper eyelid. In this report,

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we shall describe the characteristic features and incidence of the blepharoptosis of myxedema and discuss its significance and pathogenesis.

SUBJECTS AND METHODS

The eyelid signs in 45 consecutive patients with frank myxedema observed by the Endocrine Service at Cook County Hospital during 1960 were evaluated by the authors (Table 1). Thirty-two women and 9 men had primary myxedema. Their ages varied from 16 to 81 years. The duration of myxedema varied from four months to sixty-five years. The myxedema followed subtotal thyroidectomy in 8 patients, and therapy with I^{131} in 9 patients; it was idiopathic in 24 patients. In addition, a 10-month-old cretin girl, and 2 women and 1 man with myxedema secondary to hypopituitarism (craniopharyngioma, postpartum pituitary necrosis, and idiopathic respectively) were studied. Twenty-seven

TABLE 1. EYE FINDINGS* IN MYXEDEMA

Patient		Etiology of myxedema	Duration	Thyroidal I^{131} uptake (% dose)	Blepharoptosis	Tarsal fold	Voluntary & conjugate lid elevation	Pupil	Lid edema
No.	Age & sex								
				Untreated Myxedema					
1	77 F	Idiopathic	65 yrs.	5	+	+	+	N	0
2	57 F	Idiopathic	20 yrs.	2	+	+	+	N	+
3	50 F	Idiopathic	1 yr.	1	+	+	+	N	0
4	47 F	Postop.†	4 yrs.	5	+	+	+	N	+
5	66 F	Post-I^{131}‡	5 yrs.	0	+	+	+	N	+
6	47 F	Post-I^{131}	4 mos.	5	+	+	+	N	0
7	60 M	Idiopathic	12 yrs.	11	+	+	+	N	0
8	72 F	Idiopathic	8 yrs.	0	+	+	+	N	+
9	72 F	Idiopathic	20 yrs.	0	+	+	+	N	+
10	24 F	Pituitary necrosis	2 yrs.	0	+	+	+	N	+
11	44 F	Post-I^{131}	2 yrs.	0	+	+	+	N	+
12	73 F	Postop.	12 yrs.	6	+	+	+	N	+
13	70 M	Idiopathic	10 yrs.	1	+	+	+	N	+
14	32 F	Postop.	1 yr.	2	+	+	+	N	0
15	72 F	Idiopathic	10 yrs.	1	+	+	+	N	+
16	62 F	Idiopathic	10 yrs.	1	+	+	+	N	+
17	72 F	Idiopathic	10 yrs.	2	+	+	+	N	+
18	81 F	Idiopathic	19 yrs.	1	+	+	+	N	+
19	49 F	Idiopathic	7 mos.	10	+	+	+	N	+
20	54 F	Post-I^{131}	4 mos.	11	+	+	+	N	+
21	50 M	Idiopathic	3 yrs.	8	0	+	+	N	+
22	60 M	Idiopathic	12 yrs.	11	0	+	+	N	+
23	16 F	Idiopathic	8 yrs.	0	0	+	+	N	+
24	10 mos. F	Idiopathic	10 mos.	0	0	+	+	N	+
25	74 M	Postop.	9 yrs.	2	0	+	+	N	0
26	66 F	Idiopathic	2 yrs.	7	0	+	+	N	0
27	50 M	Idiopathic	2 yrs.	0	0	+	+	N	+
				Treated Myxedema					
1	69 M	Post-I^{131}	5 yrs.	5	+	+	+	N	0
2	64 F	Idiopathic	5 yrs.	2	+	+	+	N	0
3	46 M	Hypopituitary	5 yrs.	5	+	+	+	N	0
4	58 F	Idiopathic	10 yrs.	1	+	+	+	N	0
5	53 F	Craniopharygioma	2 yrs.	12	+	+	+	N	+
6	56 F	Post-I^{131}	3 yrs.	2	+	+	+	N	+
7	46 F	Post-I^{131}	6 yrs.	6	0	+	+	N	+
8	66 F	Idiopathic	10 yrs.	2	0	+	+	N	+
9	74 M	Postop.	6 mos.	2	0	+	+	N	0
10	69 F	Postop.	43 yrs.	2	0	+	+	N	0
11	55 F	Postop.	8 yrs.	2	0	+	+	N	0
12	54 F	Post-I^{131}	4 yrs.	3	0	+	+	N	+
13	57 F	Post-I^{131}	2 yrs.	4	0	+	+	N	0
14	59 F	Idiopathic	3 yrs.	2	0	+	+	N	0
15	56 M	Idiopathic	2 yrs.	6	0	+	+	N	0
16	36 F	Postop.	7 yrs.	0	0	+	+	N	0
17	41 F	Idiopathic	3 yrs.	2	0	+	+	N	0
18	42 F	Idiopathic	10 yrs.	2	0	+	+	N	0

* + =present. 0 =absent. N =normal.
† Following subtotal thyroidectomy.
‡ Following therapy with I^{131}.

patients were evaluated before, and 18 after, institution of replacement therapy with desiccated thyroid.

Blepharoptosis was defined as the extension of the upper eyelid over the iris to a point lower than 2 millimeters below the upper limbus, which is the lower limit of normal (6). If the iris is taken as the face of a clock, the upper lid margin is normally at one o'clock and no lower than two o'clock (Fig. 1a).

Following examination of the eyelids for ptosis, two to three drops of phenylephrine (Neosynephrine) hydrochloride ophthalmic solution, 2.5 or 10.0 per cent, were instilled in the conjunctival sac of one eye, and the eyelids re-examined five to fifteen minutes later.

Electromyographic studies of the levator palpebrae superioris muscle were made in 4 patients, using a clinical model electromyograph (Meditron Model 201A) with a mono-

TABLE 2. EYE FINDINGS ASSOCIATED WITH BLEPHAROPTOSIS NOT DUE TO MYXEDEMA

Patient No.	Diagnosis	Degree* of blephar-optosis	Tarsal fold	Voluntary & conjugate lid elevation	Pupil†	Response to phenylephrine
1	Myasthenia gravis	++	absent	slight	N	minimal
2	Myasthenia gravis	++	absent	slight	N	none
3	Myasthenia gravis	++	absent	absent	N	none
4	Congenital ptosis	++	absent	absent	N	none
5	Congenital ptosis	++	absent	absent	N	none
6	III nerve palsy	++	absent	absent	D	none
7	III nerve palsy	++	absent	absent	D	none
8	Horner's syndrome	+	present	present	M	present
9	Horner's syndrome	+	present	present	M	present

* ++ = marked; + = slight.
† N = normal; D = dilated; M = miotic.

polar needle electrode. Action potentials were also obtained from the biceps brachii muscle.

In 6 patients with myxedema a solution of epineprhine in physiologic saline was infused intravenously at rates varying from 0.05 to 0.40 μg. per Kg. per minute, and the response of the pupils and eyelids recorded. The 24-hour urinary excretion of catecholamines was determined in 4 patients by a modification of the fluorometric method of Lund (7).

The effects of instillation of phenylephrine hydrochloride in the conjunctival sac were also studied in 50 patients with diseases other than myxedema and without blepharoptosis (control subjects) and in 9 patients without myxedema but with blepharoptosis of other etiologies (Table 2). In 7 control subjects, a solution of epinephrine was infused intravenously at rates varying from 0.05 to 0.20 μg. per Kg. per minute.

In a corollary study, the effects of instillation of phentolamine (Regitine) methanesulfonate in the conjunctival sac were observed in 3 patients with eyelid retraction due to hyperthyroidism.

RESULTS

Blepharoptosis was observed in 20 of 27 patients (74 per cent) with myxedema evaluated before the institution of therapy. It was noted in only 6 of 18 patients (33 per cent) with myxedema first examined after therapy was begun. Characteristically, the blepharoptosis of myxedema

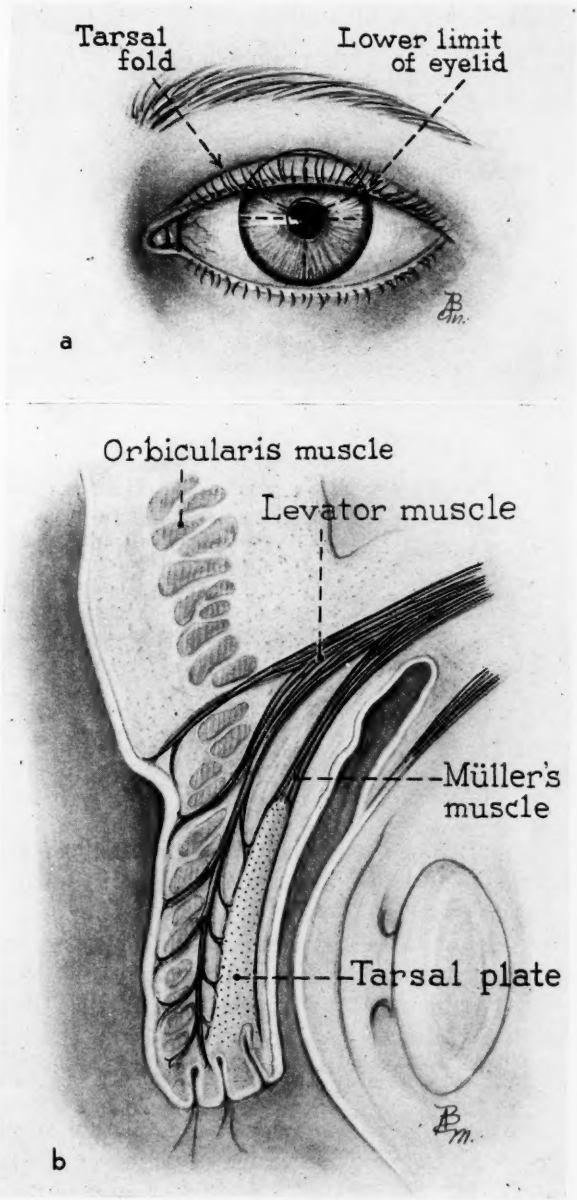

FIG. 1. **a)** Eye, showing normal lower limit of eyelid in relation to iris on forward gaze. Blepharoptosis is considered to be present when the upper lid is lower than shown. **b)** Schematic cross-section showing anatomy of upper eyelid.

was of modest degree and could best be recognized when the patient's gaze was fixed directly ahead. There was a distinct tendency to compensate for the ptosis by wrinkling the forehead. The tarsal fold was preserved, pupil size and reaction were normal, voluntary lid elevation was intact, and normal conjugate lid elevation occurred with upward gaze. As a result of the blepharoptosis, the palpebral fissure appeared narrow. In an occasional instance, slight elevation of the lower lid contributed to the narrowing of

FIG. 2. Typical examples of the blepharoptosis of myxedema. Note the moderate degree of ptosis and the persistence of the tarsal fold.

the palpebral fissure. Although usually bilateral, blepharoptosis was on occasion unilateral. Figures 2–4 illustrate representative examples of the blepharoptosis of myxedema.

Blepharoptosis could be distinguished easily from pseudoptosis due to folds of edematous skin hanging over the lid margin (Fig. 3b). Lid edema, although frequent, was not always associated with true blepharoptosis. Nine of 26 patients (35 per cent) with blepharoptosis had no lid edema, and 8 of 25 patients (32 per cent) with lid edema had no blepharoptosis (Table 1).

All patients with myxedema responded to instillation of phenylephrine into the conjunctival sac by a noticeable elevation of the upper eyelid, which characteristically returned to a normal or even retracted position, thereby emphasizing the degree of antecedent ptosis (Figs. 3 and 4). In a few instances a slight degree of retraction of the lower lid occurred. Mydriasis regularly accompanied the lid response.

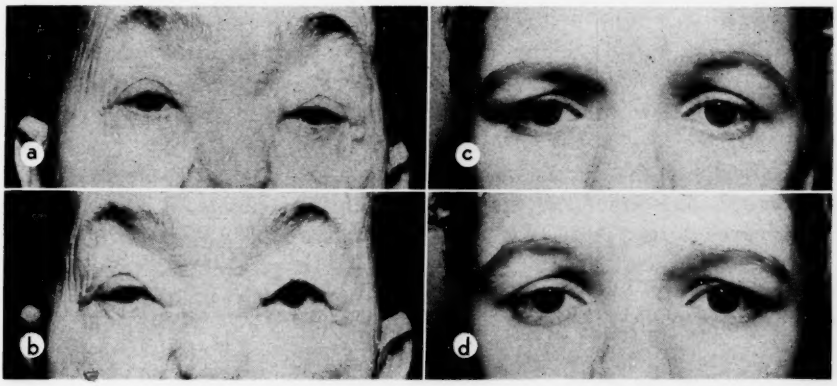

FIG. 3. Eyelid findings in myxedema before and after instillation of phenylephrine ophthalmic solution into the conjunctival sac of the left eye. Upper panel (**a** and **c**): before phenylephrine. Lower panel (**b** and **d**): after phenylephrine. Note the distinct elevation of the lid margin in each instance. In the patient on the left, pseudoptosis is evident after phenylephrine owing to edema of the lid which now overhangs the lid margin.

In 48 of 50 control subjects, distinct retraction of the upper eyelid and mydriasis occurred in response to instillation of phenylephrine.

Seven patients with myasthenia gravis, oculomotor nerve paralysis, or congenital ptosis showed marked blepharoptosis, wrinkling of the forehead

FIG. 4. Eyelid findings in myxedema before and after instillation of phenylephrine ophthalmic solution into the conjunctival sac. Upper panel: before instillation, showing ptosis of both eyes. Middle panel: after phenylephrine instillation into the left eye, showing distinct lid elevation. Lower panel: after phenylephrine instillation into both eyes, showing correction of the ptosis.

on forward gaze and, in the severest cases, compensatory dorsiflexion of the head. The tarsal fold, and voluntary and conjugate lid elevation were absent. Patients with oculomotor nerve paralysis had dilated pupils. In this group there was no eyelid response to the instillation of phenylephrine (Table 2).

The eyelid findings in 2 patients with Horner's syndrome were like those of myxedema; however, in contrast, the pupils were miotic. The response of the eyelids to phenylephrine was similar to that noted in myxedema (Table 2).

FIG. 5. The effect of instillation of phentolamine methanesulfonate into the left conjunctival sac upon eyelid retraction in hyperthyroidism. Upper panel: before phentolamine. Lower panel: after phentolamine. Note the dramatic correction of the lid retraction on the treated side. Conjunctival reaction is also evident.

Electromyograms of the levator palpebrae superioris muscle in 4 patients with myxedema uniformly were normal, whereas those of the biceps brachii muscle were typical of myxedema (8).

In 6 patients with myxedema and in 7 control subjects, intravenous infusion of epinephrine to the limit of cardiovascular tolerance had no effect upon the position of the upper eyelid or upon pupil size.

In 4 patients with myxedema, the urinary excretion of catecholamines varied from 20 to 80 μg. per twenty-four hours (normal for our laboratory).

Instillation of phentolamine methanesulfonate into the conjunctival sacs in 3 patients with hyperthyroidism resulted in dramatic correction of the lid retraction in one (Fig. 5), but no significant lid effects in the others. The irritation of the conjunctiva produced in each patient discouraged further testing.

DISCUSSION

Three factors were considered as possible causes of blepharoptosis in myxedema: 1) the mechanical effects of lid edema; 2) a neuromuscular dysfunction of the levator palpebrae superioris muscle; and 3) a neuromuscular dysfunction of Müller's palpebral muscle.

Lid edema, which is common in untreated myxedema, was found in only 65 per cent of the cases with blepharoptosis. Conversely, blepharoptosis was found in only 68 per cent of the cases with lid edema. Because of the lack of a constant association between lid edema and ptosis, lid edema cannot be considered the cause of blepharoptosis in myxedema.

Involvement of one of the two neuromuscular units which elevate the upper eyelid seems a more likely cause of blepharoptosis. Figure 1b shows the two muscles in diagrammatic cross-section. The major muscle is the levator palpebrae superioris, a striated muscle innervated by the superior division of the oculomotor nerve. It originates from the apex of the orbit and is inserted into the skin of the upper lid and anterior tarsal plate. The minor muscle is Müller's superior palpebral muscle, a small smooth muscle innervated by sympathetic nerves. It originates in the fleshy portion of the levator muscle and its insertion is on the superior border of the tarsal plate. In the lower lid, there is a comparable smooth muscle, Müller's inferior palpebral muscle. When both the levator and superior palpebral muscles are intact and normally innervated, there is no ptosis, voluntary lid elevation is possible, conjugate lid elevation occurs with upward gaze, and there is a tarsal fold, *i.e.*, a fold in the skin of the upper eyelid on forward gaze. When neuromuscular dysfunction of the levator palpebrae superioris muscle occurs, there is marked ptosis, absence of voluntary lid elevation, failure of conjugate elevation, and loss of the tarsal fold. On the other hand, when dysfunction of Müller's muscle occurs, there is only slight ptosis, while voluntary and conjugate lid elevation are normal and the tarsal fold persists. Compensatory wrinkling of the forehead on forward gaze may occur with ptosis of either type.

All patients with blepharoptosis and myxedema had lid findings characteristic of dysfunction of the sympathetic neuromuscular unit. Phenylephrine, which corrects ptosis caused by dysfunction of the sympathetic (*e. g.*, Horner's syndrome) but not that caused by dysfunction of the levator muscle, corrected the ptosis of myxedema. Electromyograms of the levator muscle were normal in myxedema. Thus, the evidence supports the conclusion that the blepharoptosis of myxedema is the result of dysfunction of the sympathetic neuromuscular unit. It is not clear whether the neural or the muscular component is primarily at fault, but the prompt response to phenylephrine and the apparent reversibility of the condition by treatment tends to favor involvement of the neural component. The

neural abnormality may be considered to be a relative decrease in sympathetic tone.

There is a body of evidence attesting to a relation between the thyroid hormone and the sympathetic catecholamines. The calorigenic, metabolic and hemodynamic action of the sympathetic catecholamines is increased by thyrotoxicosis or by administration of thyroid hormone (9–17). Brewster *et al.* (17) concluded that the manifestations of thyrotoxicosis result from augmentation of the effects of epinephrine and norepinephrine by thyroid hormone. Increased sensitivity to exogenous epinephrine has been utilized as a diagnostic test of thyrotoxicosis (18, 19). Conversely, blockade of the sympathetic system inhibits many of the actions of thyroid hormone (17, 20–22), and the effects of exogenous sympathetic catecholamines are diminished or abolished in spontaneous or experimental hypothyroidism (9, 10, 13–15, 17, 23–25). Thus these tyrosine derivatives appear to be additive or synergistic, for the effect of one is to augment the effects of the other, and in the absence of one, the effects of the other are dampened or absent.

The normal catecholamine excretion in hypothyroid patients with blepharoptosis suggests that the postulated diminished sympathetic tone of Müller's muscle is not due to insufficient circulating catecholamines, but rather reflects the deficiency of thyroid hormone. The failure of intravenously administered epinephrine to correct blepharoptosis or produce mydriasis was probably the result of insufficient elevation of the circulating catecholamines. The instillation of phenylephrine, on the other hand, probably achieved far higher local concentrations and thus effected a response.

If blepharoptosis in myxedema results from diminished tone of the sympathetic system, it is reasonable to conclude that eyelid retraction in hyperthyroidism results from excessive tone. Such an explanation has been postulated previously (26, 27). In keeping with this hypothesis, it is noteworthy that reserpine, which acts by depleting body stores of catecholamines (28), can correct lid retraction in hyperthyroidism (29), and that phentolamine, which blocks circulating catecholamines (30), can abolish lid retraction when instilled into the conjunctival sac as it did in one of our patients with thyrotoxicosis.

Other explanations for the blepharoptosis of myxedema are possible. These include reversible pathologic changes of the midbrain, of the sympathetic nerve fibers, or of Müller's muscle. However, there is little evidence to support these possibilities.

The present study indicates that blepharoptosis occurs with great regularity in patients with myxedema. It may be readily overlooked unless one is aware of its occurrence. Indeed, it appears to have escaped recognition

for years, since approximately 75 per cent of the photographs of patients with myxedema in many standard textbooks demonstrate blepharoptosis without specific mention of the sign in the accompanying text.

Blepharoptosis in myxedema appears to be the converse of lid retraction in hyperthyroidism and is similarly useful as a clinical sign. Ptosis associated with intact voluntary and conjugate lid elevation and a persistent tarsal fold should lead one to suspect myxedema even when other clinical manifestations of the disease are not overt. Blepharoptosis in myxedema is apparently a manifestation of diminished tone of the sympathetic nervous system and further attests to the interaction of thyroid hormones and the catecholamines.

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THE METABOLISM OF CORTISOL-4-C^{14} IN YOUNG AND ELDERLY MEN. I. SECRETION RATE OF CORTISOL AND DAILY EXCRETION OF TETRAHYDROCORTISOL, ALLOTETRAHYDROCORTISOL, TETRAHYDROCORTISONE AND CORTOLONE (20α AND 20β)*

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ABSTRACT

The average 24-hour secretion rate of cortisol, as determined from the excretion of any one of the four major urinary metabolites, THF, ATHF, THE, or cortolone (20α and 20β), for 8 elderly men was 75 per cent of that for 8 young men. When the secretion rates were expressed as mg. per gm. of creatinine per twenty-four hours, the average rates were essentially the same—14 mg. versus 15 mg., for both groups of subjects. These findings substantiate those obtained for the mean total 24-hour excretions of THF, ATHF, THE and cortolone, which for 23 elderly men was approximately 70 per cent of that for the 23 young men. When these values are expressed as mg. per gm. of creatinine, the mean of 4.9 mg. for young men compares with that of 4.7 mg. for elderly men. In both age groups there were essentially similar proportions of the two 11-hydroxyl (THF, ATHF) to the two 11-ketone substituted metabolites (THE, cortolone), and of the three 5β (THF, THE, and cortolone) to the one 5α (ATHF) cortisol metabolite. Aging, by the criteria employed, appears to be differentiated only by the quantity of adrenally secreted cortisol which may be related to the muscle mass of the subject.

IN PREVIOUS publications (1–4) we have presented data on the excretion of the major urinary metabolites of cortisol-tetrahydrocortisone (3α,17α,21-trihydroxypregnane-11,20-dione) (THE), tetrahydrocortisol (3α,11β,17α,21-tetrahydroxypregnane-20-one) (THF), allotetrahydrocortisol (3α,11β,17α,21-tetrahydroxyallopregnane-20-one) (ATHF), and cortolone (20α and 20β) (3α,17α,20,21-tetrahydroxypregnane-11-one)—by young and elderly men and women. Aging appeared to have the effect of reducing the 24-hour excretion of these adrenal steroids. When this

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excretion was expressed as a function of creatinine excretion, the quantitative difference between the young and the elderly disappeared. In addition, the proportions of the individual metabolites in relation to their total output did not differ significantly in young and elderly men, although there was a tendency for the latter to excrete a somewhat larger proportion of the 11-hydroxylated metabolites, THF and ATHF. The data suggested therefore that there was no essential difference due to age in the way cortisol was metabolized or in the amount of it secreted by the adrenal gland when the muscle mass of the subject was taken into consideration. However, to confirm the foregoing conclusion fully, or to prove otherwise, the adrenal secretion rates of cortisol (5–9) were determined for 8 young men and 8 elderly men after administration of tracer doses of cortisol-4-C^{14}. Quantitative estimations were also made of the major adrenal metabolites in order to determine the relationship of their excretion to the secretion rate of cortisol.

SUBJECTS AND METHODS

Subjects

The 8 young men were laboratory personnel, aged 21–35 years. The 8 elderly men, aged 65–73 years, were residents of a home for the aged and were in reasonably good health.

Methods

Approximately 1 μc. of cortisol-4-C^{14} equivalent to .046 mg. was dissolved in 0.1 ml. of ethanol and 10 ml. of physiologic saline and injected[1] into the subject's anticubital vein. Suitable aliquots for determination of radioactivity of the cortisol-4-C^{14} solution were taken prior to injection. After injection, aliquots of the solution remaining in the injection flask were again assayed for radioactivity. In addition the injection syringes were rinsed with water and aliquots of an ethyl extract of the aqueous solution were counted for radioactivity. The difference between the original radioactive content and the radioactivity recovered after injection constituted the dose of cortisol-4-C^{14}.

The subject voided and discarded a urine sample just prior to the time of injection. Urine was then collected for two 24-hour periods. Creatinine values were determined for each sample. Only results with the first 24-hour collection will be described here. Aliquots were removed from the 24-hour urine sample to determine the recovery of the injected radioactivity. These samples were counted in a Tricarb liquid scintillator, using the method described by Flood *et al.* (8).

The urine sample was incubated for twenty-four hours at 37° C. with β-glucuronidase (Sigma). After glucuronidase hydrolysis the sample was subjected to continuous extraction with ethyl acetate and washed with water and 0.1*N* NaOH as previously described (3, 4). Immediately after the alkali washes were completed, the combined 0.1*N* NaOH washes were brought to pH 7.0, followed by continuous extraction for six hours with ethyl acetate. The ethyl acetate extract was washed with water which was backwashed with ethyl acetate.

Aliquots were taken from the solvent extracts of both the neutral fraction and the NaOH washes for measurement of radioactive content with a Tracerlab SC-50 auto-

[1] Cortisol-4-C^{14} was obtained from the Endocrine Study Committee, U. S. Public Health Service.

matic flow counter using the method of infinite thin samples. All subsequent determinations of radioactivity were made in this way.

Two aliquots, each equivalent to one-third or approximately an eight-hour portion of the 24-hour sample, were taken for paper chromatography. The initial separation on paper was made in the benzene-formamide system for a period of seventy-two hours (1). Subsequently the dry paper chromatograms were placed in a chromatogram scanner (Atomic Accessories) for delineation of the distribution of the radioactive metabolites of cortisol-4-C^{14}. Two 1-mm. strips were cut lengthwise from the center of the strips for glycerol (10) and blue tetrazolium (BTZ) tests. Standard strips containing THF, THE, and occasionally 20β-cortol and 20β-cortolone, were also tested. The areas of the paper chromatogram of aliquot #1 were eluted with methanol as follows: origin, cortol, cortolone, THF, ATHF and THE, and that below THE. The radioactivity of each eluate and that of the run-off was determined. Aliquot #2 was treated in the same way, except that the area of the strip above THF, including the origin, was eluted in its entirety. Determinations of Porter-Silber (PS) chromogens were made on aliquots of THF, ATHF and THE.

The "origin," cortol, and cortolone eluates of aliquot #1 and the eluates of the total area above THF of aliquot #2 were each re-run in the chloroform system for forty-eight hours. β-Cortolone and cortolone, as well as β-cortol and cortol, do not separate from each other in this system. THF, ATHF and THE eluates were each run in the same system for twenty-four hours. The radioactivity patterns were again determined with the chromatogram scanner. Then 1-mm. strips of the chromatograms were tested with the appropriate color reagent for location of the metabolites.

For the determination of the 24-hour excretion of THF, ATHF and THE, and of the secretion rate of cortisol as determined from the eluted THF, ATHF and THE, suitable aliquots of two different concentrations in duplicate were taken for PS determination. From the same solution, appropriate aliquots were similarly taken for determinations of radioactivity. By correcting the PS values for hours of collections, the volume of urine used for extraction, the volume of ethyl extract applied to the paper, the aliquots removed for various tests and counting, and the recovery from paper chromatograms, the excretion per twenty-four hours was determined. By dividing the radioactive count per sample of metabolite by the PS content in mg. per sample of metabolite, the specific activity in c.p.m. per mg. was obtained. The secretion rate of cortisol was calculated by dividing the radioactive dose by the specific activity of THF, ATHF and THE respectively.

The cortolone and, in a few instances, the cortol areas of the chloroform-formamide chromatograms were eluted and aliquots removed for determination of radioactive content. These eluates, containing both 20α and 20β isomers, were oxidized with periodic acid (11) to obtain the respective 17-ketosteroid derivatives, 11-keto-etiocholane-3α-ol-17-one (11-KE) or 11β-hydroxyetiocholan-3α-ol-17-one (11-OHE), as follows:

One ml. of absolute methanol (A.R., acetone free) and 0.3 ml. of a periodic acid (A.R.) solution (0.05 *M* in 0.3*N* H_2SO_4) were added to the nitrogen-dried eluate. The reaction mixture was allowed to stand at room temperature for one hour. The methanol solution was transferred to a 50-ml. separatory funnel with 3×10 ml. of redistilled, peroxide-free ethyl ether (after treatment with ferrous sulphate). The ether solution was washed (3×5 ml.) with a 10 per cent sodium hydrosulfite (Mallinckrodt) solution in 0.5*N* NaOH. The hydrosulfite solution was prepared just prior to use. The ether extract was washed three times with 5 ml. of water, transferred quantitatively to an appropriate vessel and evaporated to dryness under nitrogen. Girard separation was omitted.

The oxidation products, obtained in the foregoing manner, were applied to Whatman

#540 paper strips wet with methanol:formamide, 1:1, and separated in the benzene-formamide system in four to five hours. 11-KE and 11-OHE were used as standards. The paper chromatograms were dried, scanned, and 1-mm. strips taken for a Zimmermann color test. The 11-KE or 11-OHE was eluted and the radioactive content determined. Quantitative Zimmermann determinations were also made on suitable aliquots, and expressed in terms of standard 11-KE or 11β-OHE as the case may have been. These determinations allow, as do those for the α-ketols, for the quantitative estimation of the 24-hour excretion of cortolone (20β-cortolone and 20α-cortolone) and cortol (20β-cortol and 20α-cortol), and of the specific activity of these compounds. From the latter the secretion rate of cortisol could also be determined.

RESULTS AND DISCUSSION

Recovery of radioactivity

Tables 1 and 2 summarize the urinary metabolism of cortisol-4-C^{14} in twenty-four hours by 16 young and elderly men. For both groups of sub-

TABLE 1. THE RECOVERY AND DISTRIBUTION OF RADIOACTIVITY FROM INTRAVENOUSLY ADMINISTERED CORTISOL-4-C^{14}, THE SPECIFIC ACTIVITIES OF CORTISOL METABOLITES, AND THE SECRETION RATES OF CORTISOL IN 24-HOUR URINE SAMPLES FROM 8 YOUNG MEN

Subject and age		A-21	C-23	K-23	F-25	D-30	FL-31	M-32	P-35	Mean
Total hrs. of urine collection		24.0	24.3	23.6	23.6	24.0	23.5	24.0	22.8	
Creatinine (gm./24 hrs.)		1.61	1.57	1.65	2.34	1.80	1.72	1.90	1.48	1.76
Dose of cortisol-4-C^{14} (μc.)		.92	.90	.90	.89	1.02	.92	.91	.82	
% Recovery of dose in urine		94	94	93	87	90	84	79	88	89
% Recovery of dose	in ETAC extract	55	42	46	45	57	52	52	42	49
	in NaOH extract	3	5	4	4	5	3	4	4	4
% Recovery of dose after paper chromat.	Of ETAC extract	57	43	49	45	59	47	51	49	50
	of NaOH	3	4	3	4	5	2	3	3	3
% Distribution of radioactivity after paper chromat.	more polar than THF	45	35	44	54	32	46	23	59	42
	THF, ATHF, THE	42	51	46	25	52	33	56	23	41
	less polar than THE	13	14	10	21	16	21	21	18	17
Specific activity (μc./mg.)	Cortol	.039	—	—	—	—	.059	—	—	—
	Cortolone	.038	.037	.040	—	.050	.056	.031	.029	
	THF	.038	.032	.040	.045	.054	.059	.029	.030	
	ATHF	.038	.033	.037	.042	.052	.058	.031	.031	
	THE	.038	.033	.043	.044	.050	.059	.029	.029	
	Mean	*.038*	*.034*	*.040*	*.044*	*.052*	*.058*	*.030*	*.029*	
Secretion rate of cortisol (mg./24 hrs.)	Cortol	23.6	—	—	—	—	15.5	—	—	
	Cortolone	24.0	24.2	22.8	—	20.5	16.2	29.1	28.2	
	THF	24.0	28.2	22.4	19.6	18.9	15.6	32.7	27.9	
	ATHF	24.5	27.1	24.3	21.0	19.7	15.8	29.3	27.0	
	THE	24.3	27.5	21.1	20.4	20.3	15.5	31.4	28.8	
	Mean	*24.2*	*26.8*	*22.7*	*20.3*	*19.9*	*15.9*	*31.1*	*28.0*	*23.6*
Secretion rate of cortisol (mg./gm. creatinine)	*Mean*	*15.0*	*17.1*	*13.8*	*8.7*	*11.1*	*9.2*	*16.4*	*18.9*	*13.8*

TABLE 2. THE RECOVERY AND DISTRIBUTION OF RADIOACTIVITY FROM INTRAVENOUSLY ADMINISTERED CORTISOL-4-C^{14}, THE SPECIFIC ACTIVITIES OF CORTISOL METABOLITES, AND THE SECRETION RATES OF CORTISOL IN 24-HOUR URINE SAMPLES FROM 8 ELDERLY MEN

Subject and age		G-65	H-67	A-69	M-70	P-70	L-72	LI-72	GA-73	Mean
Total hrs. of urine collection		23.5	21.5	24.0	23.8	24.0	23.9	23.8	23.0	
Creatinine (gm./24 hrs.)		1.21	1.09	1.29	1.20	1.42	1.39	0.76	1.22	1.20
Dose of cortisol-4-C^{14} (μc.)		.91	.98	.79	.15	.89	.91	.87	.73	
% Recovery of dose in urine		84	87	80	94	94	94	96	85	89
% Recovery of dose	in ETAC extract	49	53	46	52	50	51	55	53	51
	in NaOH extract	3	4	5	10	4	5	5	4	5
% Recovery of dose after paper chromat.	of ETAC extract	50	58	45	52	51	52	48	60	52
	of NaOH extract	3	5	4	6	3	5	4	4	4
% Distribution of radioactivity after paper chromat.	more polar than THF	45	44	42	34	31	51	39	38	40
	THF, ATHF, THE	48	45	46	49	56	34	43	44	46
	less polar than THE	7	11	12	17	13	15	18	18	14
Specific activity (μc./mg.)	Cortol	—	.059	—	—	.043	—	—	—	
	Cortolone	.039	.060	.039	.0098	.044	.056	.062	.060	
	THF	.043	.060	.036	.0095	.039	.056	.061	.059	
	ATHF	.038	.058	.033	.0097	.040	.054	.060	.059	
	THE	.038	.059	.033	.0097	.045	.057	.061	.058	
	Mean	*.040*	*.059*	*.035*	*.0097*	*.042*	*.056*	*.061*	*.059*	
Secretion rate of cortisol (mg./24 hrs.)	Cortol	—	16.8	—	—	21.0	—	—	—	
	Cortolone	23.1	16.4	19.8	15.2	20.1	16.1	14.0	12.1	
	THF	21.2	16.3	21.5	15.9	23.0	16.3	14.4	12.3	
	ATHF	24.1	17.0	23.4	15.5	22.1	16.7	14.6	12.4	
	THE	23.7	16.8	23.4	15.4	19.8	15.9	14.3	12.5	
	Mean	*23.0*	*16.7*	*22.0*	*15.5*	*21.3*	*16.3*	*14.3*	*12.3*	*17.7*
Secretion rate of cortisol (mg./gm. creatinine)	*Mean*	*19.0*	*15.3*	*17.1*	*12.9*	*15.0*	*11.7*	*18.8*	*10.1*	*15.0*

jects the recoveries of radioactivity in the urine ranged from 79 to 96 per cent, with an average recovery of 89 per cent of the dose. These values compare with those obtained for 5 subjects by Flood *et al.* (8), namely, 83–96 per cent, with a mean recovery of 90 per cent. In the crude ethyl acetate extract obtained after glucuronidase hydrolysis, the mean recovery was 50 per cent of the dose for young subjects and 52 per cent for elderly subjects. These values would presumably be higher in view of the self-adsorption of radioactivity encountered in particularly pigmented urine extracts. The crude ethyl acetate extracts of the alkali washes accounted for 3 per cent and 4 per cent, respectively, of the radioactive dose.

The recoveries of radioactivity after paper chromatography of the crude ethyl acetate extract in the benzene-formamide system were as follows: 43 to 60 per cent (mean, 51 per cent) for young men, and 45 to 60 per cent (mean, 52 per cent) for the elderly men; 3 per cent and 4 per cent, respec-

tively, were the proportions of the radioactivity recovered after paper chromatography in the benzene-formamide system of the ethyl acetate extract of the alkali washes.

Essentially no self-adsorption of radioactivity was observed in counting the aliquots of eluates from the benzene-formamide paper chromatograms. However a 5 to 15 per cent loss of metabolites, and consequently of radioactivity, was sustained as the result of this procedure. Presumably then our recoveries would be approximately 10 per cent greater. A mean recovery of 56 per cent would compare with that of 55 per cent obtained by Fukushima *et al.* (12) and with that of 62 per cent obtained by Flood *et al.* (8).

Distribution of radioactivity

Tables 1 and 2 list the data on the distribution of radioactivity of the glucuronidase-hydrolysis urinary extracts in the benzene-formamide paper chromatogram, expressed as that occurring above THF, as that including THF, ATHF and THE, and as that less polar than THE and including all radioactive metabolites from allotetrahydrocortisone (ATHE) to, and including 17-ketosteroids. The proportions of the radioactivity above THF (including cortols, cortolones, 6β-OH-cortisol and any other polar metabolites) ranged from 23 to 59 per cent with a mean of 42 per cent for young men, and from 31 to 51 per cent with a mean of 40 per cent for the elderly men; from 25 to 56 (mean, 41 per cent) and from 34 to 63 (mean, 46 per cent) were present in the THF, ATHF and THE fractions, respectively. The proportions of less polar radioactivity varied from 10 to 21 (mean, 17 per cent) for the young subjects, and from 7 to 18 (mean, 14 per cent) for the elderly subjects.

Peterson (6) previously indicated that THF, ATHF, THE and ATHE accounted for approximately 50 per cent of cortisol metabolites in the neutral steroid fraction after hydrolysis with β-glucuronidase. Fukushima *et al.* (12) found that THF, ATHF and THE amounted to 45 or 50 per cent of this extract. Our mean values of 41 per cent for the young men and 47 per cent for the older men are of a similar order. If the values in the young men (Subjects F, Fl and P) who had "atypical" patterns of cortisol metabolites are excluded, the remaining values ranged from 42 to 56 per cent (mean, 49 per cent). Flood *et al.* (8) obtained for 5 women a somewhat higher mean value of 63 per cent as THF, ATHF and THE.

The radioactivity found in the area of the paper chromatograms above THF not only comprises that from all the cortols, allocortol, cortolones and allocortolones (12) but from at least 2 BTZ-positive metabolites, one of which has an R_f similar to that for 6β-OH-cortisol (12, 13). In addition, radioactivity is regularly found in areas more polar than cortol. The mean values for this fraction—42 per cent for the young and 40 per cent for the

elderly subjects—would therefore presumably be higher than those values of 18 to 33 per cent found by Fukushima *et al.* (12) for only the reduced glycerols. Flood *et al.* projected a mean value of 33 per cent in this fraction in their study of 5 women.

Fukushima *et al.* (12) found 10 to 15 per cent of the total radioactivity in the fraction less polar than cortisone. Our less polar fraction, which also includes cortisone, cortisol and ATHE, contained 10 to 21 per cent in the young men and 7 to 18 per cent in the elderly.

Of the 3–4 per cent radioactivity from the dose of cortisol-4-C^{14}, as found in the ethyl acetate extract of the alkali washes after paper chromatography in the benzene-formamide system, 80 per cent had a mobility of, or less than that of cortol.

Specific activities and secretion rates

ATHF and THE do not separate in the benzene-formamide system and therefore require separation in the chloroform-formamide system. However, the PS values for THF in both systems are usually quite similar. The specific activities for THF are, on the other hand, excessively high after the initial chromatogram. The specific activities of THF, ATHF and THE are generally within 10 per cent of one another after chromatography in the chloroform-formamide system. Occasionally one of these metabolites has had, after chloroform-formamide separation, a specific activity greater or less than 10 per cent of the values for the other two. After a second chromatogram of all 3 metabolites in the chloroform-formamide system, the specific activities of 2 of the metabolites are found to duplicate their previous values, and the value for the third has been reduced to a similar order. This kind of discrepancy has usually been observed in extracts with excessive pigments.

The ATHF from each of 3 subjects in whose chromatograms the foregoing difficulty was encountered, was crystallized and the specific activity determined from weighed amounts of the steroid. The ATHF for Subject C had a specific activity per mg. of .036 μc.; for Subject D, .050 μc.; and for Subject M, .030 μc. These were essentially similar to the respective values obtained using colorimetry (Table 1).

The quantitative Zimmermann values obtained after the periodic acid oxidation of cortolone (20α and 20β) and prior to paper chromatography were generally 25 per cent greater than that derived from the paper-separate 11-KE. The specific activities, however, were frequently double that obtained for 11-KE after chromatography. Both observations are explained by the repeated appearance on the paper strips of three to four areas of radioactivity in addition to that for 11-KE, two or three which are more polar than 11-KE, and one, less polar. Paper chromatography of the oxida-

tion product of cortol also results in areas of radioactivity other than 11-OHE. For determination of the specific activity of cortolone or cortol by this procedure, paper separation of either 11-KE or 11β-OHE appears to be necessary.

The values for the specific activities of the various metabolites presented in Tables 1 and 2 indicate that for any given subject the values agree rather closely (*i.e.*, $\pm$ 10 per cent), and that any one metabolite might be used to determine the secretion rate of cortisol in 24-hour urine samples from normal subjects. Other investigators (6, 8, 9) have obtained similar results.

The secretion rates for the young men (Table 1) ranged from 16 to 31 mg., and for the elderly men (Table 2) from 12 to 23 mg. per twenty-four hours. These compare with those reported by Cope and Black (5), Peterson (6), Brookes and Prunty (7) and Flood *et al.* (8). When the secretion rates were expressed in terms of creatinine, the mean 24-hour secretion rate for young subjects was 14 mg. per gm. of creatinine and for elderly subjects 15 mg. per gm. These data confirm our previous findings derived from the 24-hour excretion of THF, ATHF and THE, *i.e.*, that on a 24-hour basis, young subjects have a greater output of cortisol than elderly subjects. When presumably muscle mass (creatinine) is taken into consideration, the young do not differ significantly from the elderly in their secretion of this hormone.

24-Hour excretion of cortisol metabolites

Table 3 summarizes the excretion of 4 cortisol metabolites by young and elderly men, in terms of both mg. per twenty-four hours and mg. per gm.of creatinine per twenty-four hours. The mean 24-hour values of THF (1.8 mg.), ATHF (1.4 mg.), THE (2.5 mg.) and cortolone (1.2 mg.) for the young men were comparable to values of 1.8 mg., 1.2 mg., 2.2 mg. and 1.2 mg. respectively for the elderly men. When these values were combined with similar ones obtained previously for 15 young and 15 elderly men, the mean values for 23 young subjects [1.9 mg., 1.2 mg., 3.3 mg. and 1.4 mg. (N for cortolone = 13)] for each of the four metabolites were greater than those for the 23 older men [1.5 mg., 1.0 mg., 1.9 mg. and 1.0 mg. respectively (N for cortolone = 11)]. However, the values of THF, ATHF, THE and cortolone expressed in mg. per gm. of creatinine were similar for both groups of 23 men—1.2 mg., 0.8 mg., 2.0 mg. and 0.9 mg. for the young subjects versus 1.3 mg., 0.9 mg., 1.7 mg. and 0.8 mg. respectively for the elder subjects. The mean 24-hour excretion of the 4 cortisol metabolites (5.4 mg.) by the older men was approximately 70 per cent of that (7.9 mg.) by the younger men. The mean value of 4.7 mg. per gm. of creatinine for the elder subjects was virtually the same as 4.9 mg. for the young subjects. Other workers (8, 12, 14–16) have found the 24-hour excretion of THF,

TABLE 3. EXCRETION VALUES OF THF, ATHF, THE AND CORTOLONE FOR YOUNG AND ELDERLY MEN, EXPRESSED AS MG./24 HRS. AND AS MG./GM. CREATININE

Young men		Mg. per 24 hrs.					*Elderly men*		Mg. per 24 hrs.				
		THF	ATHF	THE	Cortolone	Total			THF	ATHF	THE	Cortolone	Total
A.		1.96	1.40	2.77	1.31	7.44	*Ge.*		1.95	1.72	2.77	1.11	7.55
C.		1.54	1.51	1.98	0.97	6.00	*H.*		2.55	1.04	3.16	1.27	8.02
K.		1.34	1.11	3.28	1.29	7.02	*A.*		1.65	1.20	2.35	1.19	6.39
F.		0.94	0.65	1.41	0.98	3.98	*M.*		1.72	1.13	1.72	0.90	5.47
D.		2.77	1.60	4.38	1.21	9.96	*P.*		1.87	1.74	3.67	1.96	9.24
Fl.		0.77	1.20	0.44	0.58	2.99	*L.*		1.59	0.36	0.92	—	—
M.		3.52	2.68	3.75	1.37	11.32	*Li.*		1.59	0.78	1.23	0.75	4.35
P.		1.16	0.77	1.40	1.47	4.80	*Ga.*		1.37	1.32	1.73	—	—
	Mean	1.75	1.37	2.48	1.15	6.19		Mean	1.79	1.16	2.19	1.20	6.84
	Mean-23*	1.92	1.24	3.30	1.44†	7.90		Mean-23*	1.45	1.00	1.94	0.98‡	5.37
	Range-23*	0.8–3.5	0.4–3.1	0.4–6.3	0.3–2.9	—		Range-23*	0.3–2.6	0.3–1.7	0.7–3.7	0.6–2.0	—
		Mg. per gm. creatinine							Mg. per gm. creatinine				
A.		1.22	0.88	1.72	0.67	4.49	*Ge.*		1.61	1.43	2.29	0.92	6.25
C.		0.98	0.96	1.26	0.62	3.82	*H.*		2.34	0.95	2.90	1.17	7.36
K.		0.81	0.67	1.99	0.78	4.25	*A.*		1.28	0.93	1.82	0.92	4.95
F.		0.40	0.28	0.60	0.56	1.84	*M.*		1.43	0.94	1.43	0.75	4.55
D.		1.54	0.89	2.43	0.67	5.53	*P.*		1.32	1.23	2.58	1.38	6.51
Fl.		0.45	0.70	0.26	0.34	1.75	*L.*		1.14	0.26	0.66	—	—
M.		1.85	1.41	1.97	0.72	5.95	*Li.*		2.09	1.03	1.62	0.99	5.73
P.		0.78	0.52	0.95	0.99	3.24	*Ga.*		1.12	1.08	1.41	—	—
	Mean	1.00	0.79	1.40	0.67	3.86		Mean	1.60	1.08	2.01	1.02	5.71
	Mean-23*	1.21	0.78	2.02	0.85†	4.86		Mean-23*	1.29	0.87	1.71	0.84‡	4.71
	Range-23*	0.4–1.9	0.3–2.2	0.3–4.2	0.3–2.0	—		Range-23*	0.3–2.7	0.2–1.4	0.7–3.0	0.5–1.4	—

* Includes 15 subjects from a previous study.
† Number = 13.
‡ Number = 11.

ATHF and THE in normal subjects to be of a similar order of magnitude.

The mean ratios of THF, ATHF and THE to their sum total excretion were 32, 26 and 42 per cent respectively for the 8 young men, as indicated in Table 4. These compare with mean ratios of 37, 22 and 41 per cent for the 8 older men. The mean ratios of the 23 young men were 31, 20 and 49 per cent, and those for the 23 elder subjects were 33, 23 and 44 per cent. Older subjects have a slightly greater proportion of the 11-hydroxylated metabo-

TABLE 4. THE RATIOS OF EXCRETION OF THF, ATHF, THE AND CORTOLONE TO TOTAL EXCRETION OF THF, ATHF AND THE

Young men	THF	ATHF	THE	Cortolone	Elderly Men	THF	ATHF	THE	Cortolone
A	32	23	45	21	*Ge*	30	27	43	17
C	31	30	39	19	*H*	38	15	47	19
K	24	19	57	23	*A*	32	23	45	23
F	32	21	47	33	*M*	37	25	38	20
D	32	18	50	14	*P*	26	24	50	27
Fl	32	50	18	24	*L*	55	13	32	—
M	35	27	38	14	*Li*	44	22	34	21
P	35	23	42	44	*Ga*	31	30	39	—
Mean	32	26	42	24	*Mean*	37	22	41	21
*Mean-23**	31	20	49	24†	*Mean-23**	33	23	44	20‡

* Includes 15 subjects from a previous study.
† Number = 13
‡ Number = 11.

lites. Other investigators (8, 12, 14–16) have found similar relationships between the excretions of THF, ATHF and THE. The mean ratio of 24 per cent of the excretion of cortolone to the total excretion of THF, ATHF and THE for the 13 young subjects compares to the ratio of 20 per cent for the 11 older men.

The mean ratio of the two 11-ketonic metabolites (THE and cortolone) to the two 11-hydroxylated metabolites (THF and ATHF) was the same (53 to 47 per cent) for both groups of men. The mean ratio of the three 5β-metabolites to the one 5α-ATHF was 79 to 21 per cent for the young men and 81 to 19 per cent for the elderly.

Cortolones

In a previous publication (4) we reported the isolation and identification of 20β-cortolone from the cortolone area of paper chromatograms of urinary extracts. At that time we were unable to demonstrate the presence of 20α-cortolone, which had been isolated from urinary extracts by Fukushima *et al.* (17). We have since isolated and identified 20α-cortolone in a manner similar to that described by Fukushima *et al.* (12). A pool of extracts from the urine of normal subjects was made and chromatographed in benzene-formamide and chloroform-formamide in the usual manner. 20β-

Cortolone was crystallized in ethyl acetate-acetone from the cortolone area eluate and identified as that steroid by infrared analysis. The mother liquor was acetylated with acetic anhydride and pyridine. A substance crystallized from the acetylated mother liquor in neohexane-benzene had a melting point of 216–217°C. The known pregnane-3α,17α,20α,21-tetrol-11-one-3,20,21-triacetate (20-α-cortolone triacetate) also melted at 216–217°C. The infrared spectrum of the unknown compound was identical with that of 20α-cortolone triacetate. Presumably 20α-cortolone is present with 20β-cortolone in the cortolone area of paper chromatograms of the neutral urinary extracts. The excretion values previously ascribed to 20β-cortolone (4) therefore include both steroids.

In the current study the proportions of radioactivity in the extracts (glucuronidase hydrolysis) associated with the cortolone area after the second chromatogram in chloroform-formamide varied from 14 to 29 per cent (mean, 20 per cent) for the young men, and from 15 to 31 per cent (mean, 24 per cent) for the elderly men. These compare with 5 values of 14 to 24 per cent for the cortolone area obtained for 5 men by Fukushima *et al.* by a different system of paper chromatography. Furthermore, Fukushima *et al.* were able to account for most of this radioactivity as 20α and 20β cortolone by reverse isotope dilution techniques.

On the other hand, of the original 20 per cent of radioactivity associated with the cortolone areas in this study, a mean of 52 (33–71) per cent was accounted for as 11-KE (*i.e.*, 20α- and 20β-cortolone) for the young subjects. Of the original 24 per cent for the elderly subjects, 54 (31–72) per cent was accounted for as 11-KE. Furthermore, an inverse relationship was noted between the radioactivity before periodic acid oxidation and that recovered afterwards as 11-KE. When for a given subject the proportion of radioactivity of the neutral fraction eluted from the cortolone area after paper chromatography in the chloroform-formamide system was greater, that recovered as 11-KE was smaller, and vice versa. As a result, the means of a narrow range of values for the excretion of 20α- and 20β-cortolone as a percentage of the cortisol metabolites in the neutral fraction were 9.8 (8.6 to 11.1) per cent for the 8 young men; and 12.7 (9.6–12.7) per cent for 5, and 18.6 per cent for 1 of the 6 elderly subjects. These values are considerably lower than those obtained in 5 male subjects by Fukushima and co-workers.

The inverse relationship between the radioactivity of the cortolone area and that recovered as 11-KE can probably be explained by the fact that when the radioactivity of the former was proportionately higher after periodic acid oxidation, several areas of considerable radioactivity other than 11-KE were found on paper chromatograms of the oxidation products. When a smaller percentage of the radioactivity of the neutral extract was

present in the cortolone areas, radioactivity in areas of the paper chromatogram other than 11-KE was considerably reduced.

In an endeavor to account for the lower cortolone values, experiments for recovery of known amounts of 20α- and 20β-cortolone from paper chromatograms in both chloroform-formamide and benzene-formamide systems and periodic acid oxidation were repeated. In addition, known amounts of 20β-cortolone were added to aliquots of radioactive cortolone from paper chromatogram eluates. Recoveries of 20α- or 20β-cortolone as 11-KE in both experiments varied from 95 to 103 per cent and confirmed our previous results (4). Apparently no extensive loss of 20α- or 20β-cortolone is sustained in these procedures. The possibility exists that, after glucuronidase hydrolysis, ethyl acetate extracts a quantitatively different proportion of the various polar cortisol-4-C^{14} metabolites.

Whatever the explanation for the particular proportions of 20α- and 20β-cortolone found in these experiments, the fact that in determinations made to date on 24 young and elderly men the proportions are in a consistent correlation with THF, ATHF and THE excretion, suggests that they have a certain usefulness in assessing the relationships of the major cortisol metabolites to one another. The method for estimating cortolones has also allowed for the determination of the secretion rate of cortisol.

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THE CYCLICAL PATTERN OF RENAL EXCRETION DURING THE ADAPTATION OF NORMAL SUBJECTS TO DESOXYCORTICOSTERONE-INDUCED SODIUM RETENTION*

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ABSTRACT

The adaptation to prolonged mineralocorticoid administration was studied in 2 normal subjects receiving a constant weighed diet and daily intramuscular injections of 20 mg. of desoxycorticosterone acetate. After an initial phase of sodium retention and potassium loss (precyclical period), there was a cyclical phase characterized by depressions and elevations of urinary creatinine, osmolality, potassium, hydrogen ion, ammonia and phosphate, accompanied during the depressions by increases in free water loss, serum sodium concentration and osmolality (cyclical period). Sodium excretion increased progressively with each subsequent cyclical elevation of urinary creatinine and potassium, until levels of sodium intake were exceeded. It is postulated that this cyclical response to desoxycorticosterone-induced sodium retention is a heightening and compression of the normal 4–6 day cycle of sodium and water excretion, and that the ability to maintain or exaggerate such cycles may be a reflection of fluctuations in intrarenal hemodynamics.

NORMAL subjects adapt to exogenous aldosterone[1] or desoxycorticosterone[2] (DOC) without significant edema (1–5), and in the presence of continued administration of these salt-retaining hormones, urinary sodium excretion again becomes equal to sodium intake. Although exceptions have been reported (6–9), patients with primary aldosteronism and uncomplicated adrenal insufficiency exhibit a similar adaptation (10, 11). However, the administration of mineralocorticoid to patients with conges-

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[1] Pregn-4-ene-11β,21-diol-18-al-3,20-dione.

[2] Pregn-4-ene-21-ol-3,20-dione.

ive heart failure, nephrosis or cirrhosis usually results in excessive sodium retention and marked edema (12).

Although the mechanisms involved in the normal adaptation to salt-retaining hormone remain obscure, certain observations have been made in association with its administration:

1) An initial retention of sodium and chloride with a greater retention of sodium than chloride (3, 4, 13, 14).
2) An initial loss of potassium with production of hypokalemic alkalosis (2–4, 13, 15).
3) A later phase of increased sodium loss, and at times a decreased potassium loss (1–5).
4) An increased or unchanged serum sodium concentration (2–5, 14, 16).
5) A tendency to polyuria, with urine of low specific gravity (15, 16).
6) An initial increase in plasma and extracellular fluid volume which persists or returns to, or below control levels with a return to normal daily sodium excretion (2, 4, 17, 18).
7) An increase in total exchangeable sodium which persists despite the onset of increased sodium loss after the initial period of retention (4, 14).
8) Variable changes in endogenous creatinine clearance (1, 5, 19), and an increase in the clearance of inulin (18, 20).
9) The reduction or abolition of these effects during periods of reduced sodium in the diet (3, 4, 21).

In addition to confirming certain of these observations, the present study illustrates that an "escape" from DOC-induced sodium retention may be associated with marked cyclical depressions of urinary solute excretion prior to periods of maximal sodium loss. Such cycles are not observed in patients with marked edema who are receiving mineralocorticoid (12). The ability to maintain or amplify these cycles in the normal subject may represent a fundamental adaptive mechanism to the stress of increased volume expansion.

SUBJECTS AND METHODS

Two healthy adult males (J.F. and T.C., both aged 37) were studied during the autumn months while carrying on usual laboratory research activities but avoiding vigorous exercise. Both subjects received 20 mg. daily of desoxycorticosterone acetate intramuscularly in two doses, except as noted. They received constant weighed diets providing 2900 calories, 143 mEq. of sodium and 92 mEq. of potassium for Subject J.F. and 2167 calories, 144 mEq. of sodium and 75 mEq. of potassium for Subject T.C. Fluids were allowed without restriction. Urines were collected every twenty-four hours and at times every twelve hours, and stored at 5° C. with toluene as preservative. The subjects (fasting) were weighed daily. Serum sodium and potassium levels and osmolality were initially measured every two to three days, and subsequently twice daily at 8:00 a.m. and 6:00 p.m. before meals.

Methods of analysis have been described in recent reports from this laboratory (5). In addition, measurements were made of urinary chloride (22), ammonia (23), phosphate (24) and creatine (25). Urinary pH and titratable acidity were measured with a Beckman pH meter (26).

RESULTS

General effects

Both subjects experienced a gradual onset of muscular weakness after approximately ten days of DOC administration. This was more marked in Subject J. F., in whom there was a transient episode of paralysis on day 22 Malaise, headache, slight periorbital and ankle edema followed by thirst usually occurred in cyclical fashion, being more evident on days immediately prior to sodium and water loss and disappearing with each cycle of natriuresis. The maximal weight gain during DOC administration was 2.1 Kg. for Subject T. C., and 1.7 Kg. for Subject J. F. There were no significant changes in blood pressure.

Effect on renal patterns of creatinine and electrolyte excretion

After the initial phase of sodium retention and potassium loss induced by DOC (precyclical period), both subjects suddenly produced a dilute alkaline urine containing decreased amounts of creatinine, potassium, ammonia, hydrogen ion and phosphate but with little change in sodium and chloride content. This lasted for one day and was followed by a return of these elements to, or above previous levels. Subsequent cycles occurred in similar fashion, but each ensuing return of these substances to the values of the precyclical period was now accompanied by an increased excretion of sodium and chloride until levels of sodium intake were exceeded and the "escape" from DOC-induced sodium retention was effected. The cyclical period was considered to range from the day of initial creatinine depression to the day of maximal sodium loss. The complete data are given in Tables 1 and 2.

Creatinine excretion. During the control and precyclical periods there was no change in the daily pattern of creatinine excretion, but in both subjects a profound and surprising decrease in creatinine excretion marked the onset of the cyclical period (Figs. 1 and 3). Subsequently, urine creatinine rose and fell every one to two days in a cyclical manner that became less marked as sodium excretion increased. Unfortunately, the serum creatinine level was not determined during the periods of cyclical creatinine depression. Simultaneous determination of urine creatine demonstrated that there was no rehydration of creatinine to creatine to account for the periods of low creatinine excretion. Although not shown, the urinary creatine excretion pattern paralleled the creatinine pattern quite closely.

Sodium, potassium and chloride excretion. The precyclical period of sodium retention and potassium diuresis lasted six days in Subject J.F. and eleven days in Subject T.C. Coincident with the initial decrease in urinary creatinine, there was an abrupt fall in urinary potassium in Subject J.F. from 90 mEq. per twenty-four hours on day 12 to 33 mEq. on day 13, a decrease of 63 per cent (Fig. 1). On the following day (day 14), the 24-hour urinary potassium rose to 109 mEq. and, in parallel with the creatinine cycles, this sequence of events initiated a cyclical retention and loss of urinary potassium which became progressively less marked and disappeared entirely after the third cycle on day 23. During the initial potassium retention, urinary sodium excretion was virtually unchanged, but with each subsequent cycle of potassium loss, urinary sodium excretion rose progressively in a parallel fashion so that sodium excretion exceeded the intake by days 20 and 21. It can be seen in Figure 1 that, as the initial cyclical loss of

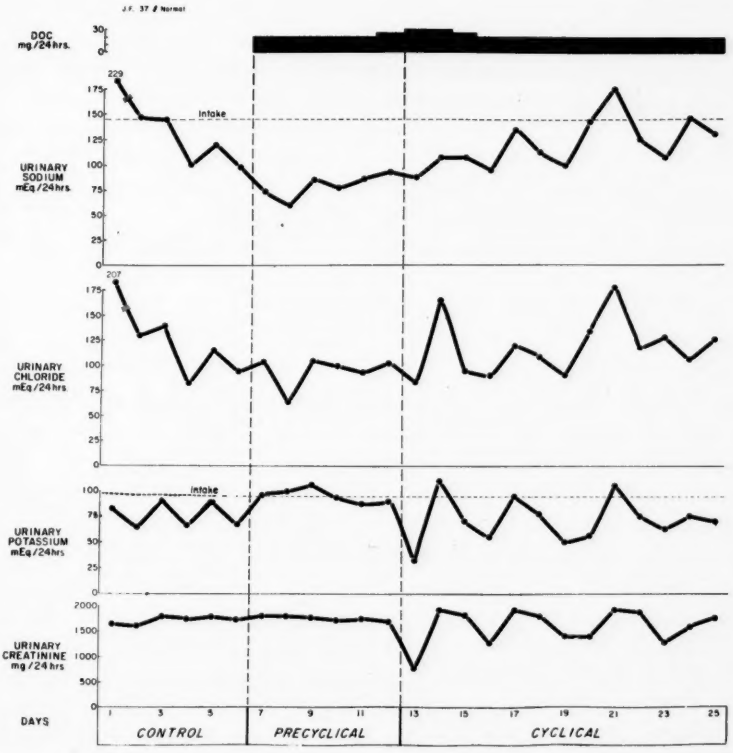

FIG. 1. The cyclical response of urinary creatinine and electrolytes induced by DOC in Subject J.F. The "escape" from sodium retention occurred on day 21.

TABLE 1. METABOLIC DATA ON SUBJECT J.F.

Day	Body wt. (Kg.)	Intake (ml./day)	Urine												Serum			DOC therapy** (mg./day)
			Output (ml./day)	Creatinine (mg./day)	Osmolality (mOsm./Kg. H_2O)	Osmotic excretion (mOsm./day)	mEq./day						pH (units)	17-Hydroxy corticoids (mg./day)	Na (mEq./day)	K (mEq./day)	Osmolality (mOsm./Kg. H_2O)	
							Na	K	Cl	T.A.*	NH_3	P						
1	71.80	1800	2520	1653	439	1106	229	82	207	27	35	58	6.45	1.8	145	4.6	292	0
2	71.05	1675	1510	1622	597	901	146	64	130	33	54	66	6.18	4.9				0
3	71.20	1900	1880	1790	543	1021	142	90	139	24	46	61	6.50	6.3	147	4.6	292	0
4	71.10	1900	1900	1729	471	895	102	65	82	33	59	53	6.19	10.5				0
5	71.20	1650	1608	1786	569	915	122	88	114	20	44	39	6.53	10.1	145	4.5	277	0
6	71.30	1800	1495	1740	568	849	99	66	90	27	45	59	6.26					0
7	71.35	1800	1690	1807	535	904	74	96	103	27	46	59	6.34	4.5				0
8	71.25	1850	1560	1816	548	855	60	100	64	26	46	72	6.50	5.5	138	4.4	272	20
9	71.55	1900	1700	1780	562	955	85	106	104	25	53	63	6.53	4.6				20
10	71.80	1850	1860	1722	493	917	77	95	100	28	51	39	6.27	7.0	142	4.3	278	20
11	72.10	1750	1580	1740	533	842	86	87	94	22	48	57	6.49	4.8				20
12	72.20	1850	1690	1690	561	948	93	90	103	24	53	51	6.53	3.7	141	4.0	281	25
13	72.20	1850	2015	758	260	524	88	33	83	4	25	15	7.02	2.2				30
14	72.30	1900	2500	1888	454	1135	107	109	165	18	59	58	6.78	2.8				30
15	72.15	1600	1510	1815	572	863	108	72	95	22	51	57	6.64	4.9	142	3.6	276	25
16	72.25	1850	2020	1242	338	683	95	54	91	9	39	31	6.88	10.8				20
17	72.10	1850	1930	1911	519	1001	135	93	120	25	61	52	6.61	6.8	142	3.8	276	20
18	72.20	1750	1510	1777	593	895	113	78	109	26	54	55	6.47	9.0				20
19	72.60	1850	1660	1374	456	757	99	51	90	12	53	37	6.77	11.2				20
20	72.75	1850	2100	1367	391	821	142	57	134	10	39	35	6.92	8.2	141	4.1		20
21	72.40	2000	2830	1916	405	1146	176	105	178	19	58	66	6.75	7.4				20
22	72.10	1750	1780	1866	522	913	125	75	118	18	59	69	6.77	5.9	141	3.7	276	20
23	72.25	1850	2010	1262	434	802	107	62	128	18	45	43	6.59	7.8				20
24	72.20	1750	1950	1536	556	973	145	74	106	22	48	69	6.64	5.4				20
25	72.15	1850	2070	1725	497	919	131	71	125	20	51	53	6.59	8.2	155	4.1	303	20
26	71.65	1950	1565	1842	625	978	119	77	130	23	54	63	6.53	1.6	142	3.7	279	20
27	71.35	1850	1450	1904	587	851	96	67	146	26	50	60	6.39	2.5	141	3.8	277	20
28	72.00	1750	1970	1458	419	825	123	69	114	10	43	42	6.92	3.4	142	3.8	280	20
29	72.05	1500	1920	1471	378	726	92	65	130	14	44	48	6.78	4.3	146	3.7	286	20
30	71.80	1550	2010	1928	548	1101	143	89	154	22	59	56	6.67	2.3	146	3.9	283	20
31	71.85	1850	2220	1747	440	977	148	77	122	16	50	40	6.79	2.7	145	4.0	289	20

* Titratable acidity.
** Desoxycorticosterone acetate in oil.

potassium became replaced by cycles of increasing sodium excretion, the net effect was an equalization of electrolyte intake and output. Thus, in this sense, there was an "escape" from continuous potassium loss as well as sodium retention.

Similar results were obtained in Subject T.C. At the time of decreased creatinine excretion, urinary potassium fell from 67 mEq. per twenty-four hours on day 17 to 42 mEq. on day 18, a decrease of 37 per cent. This was accompanied by a decrease in sodium excretion from 113 mEq. to 96 mEq. per twenty-four hours. The return of urinary potassium to precyclical values on day 20 occurred with a simultaneous increase in sodium excretion to levels exceeding intake by day 31 (Table 2).

Chloride excretion in both subjects followed the pattern of sodium excretion, but quantitatively much less chloride than sodium was retained during the precyclical period. In Subject J.F. about 73 per cent, and in Subject T.C. about 64 per cent of the chloride excreted in excess of the sodium could be accounted for by the increased potassium loss during this period. During the subsequent cyclical phase, the average daily chloride loss approximated the sodium loss.

Titratable acidity, and ammonia and phosphate excretion. These values closely paralleled the alternate depression and elevation of urinary creatinine and potassium. In Subject J.F., titratable acidity fell from 24 mEq. per twenty-four hours on day 12 to 4 mEq. on day 13, a decrease of 83 per cent (Fig. 2). At the same time ammonia excretion fell by 53 per cent and phosphate excretion by 70 per cent. Urinary pH on this day rose from 6.54 to 7.02.

In Subject T.C., the corresponding values on day 18 were a decrease in titratable acidity of 69 per cent, ammonia excretion 46 per cent, and phosphate excretion 65 per cent (Fig. 3). In both subjects, these initial depressions were restored to approximately precyclical values on the following day, but repeated cyclical depressions occurred every one to three days, exactly coinciding with the cyclical depressions of creatinine and potassium. Urinary pH showed cyclical rises corresponding to the periods of decreased titratable acidity.

Effect on urinary concentration

At the onset of cycling, when an alkaline urine with a low content of creatinine, potassium, phosphate and ammonia was produced, the osmolality of the urine fell from 561 mOsm. per Kg. of water to 260 mOsm in Subject J.F., with an increase in urine flow from 1690 ml. to 2015 ml. per twenty-four hours (Fig. 4). The total osmotic excretion fell on this day from 948 mOsm. per twenty-four hours to 524 mOsm. Since the excretion of sodium chloride was unchanged at this time, the production of a urine

TABLE 2. METABOLIC DATA ON SUBJECT T.C.

Day	Body wt. (Kg.)	Intake (ml./day)	Urine										pH (units)	Serum			DOC therapy** (mg./day)
			Output (ml./day)	Creatinine (mg./day)	Osmolality (mOsm./Kg.H_2O)	Osmotic excretion (mOsm./day)	mEq./day							Na mEq./day)	K mEq./day)	Osmolality (mOsm./Kg.H_2O)	
							Na	K	Cl	T.A.*	NH_3	P					
1	62.40	1500	1240	1397	546	677	96	54	97	28	42	42	5.68	145	3.9		-0
2	63.05	1400	1300	1505	583	757	143	59	125	21	38	33	6.18				0
3	62.65	1400	1530	1424	467	715	124	58	122	22	36	37	6.03	145	4.3	287	0
4	62.70	1400	1200	1390	578	693	123	49	107	22	30	37	6.09				0
5	62.70	1400	960	1331	705	676	122	54	69	14	32	33	6.37	143	4.0	275	0
6	62.70	1400	1280	1284	508	650	115	53	119	21	37	32	5.82				0
7	62.60	1400	1280	1487	535	685	74	79	92	20	33	37	6.18				20
8	62.55	1400	940	1467	637	599	47	74	62	22	36	31	6.12	139	4.0	272	20
9	63.05	1400	1170	1482	528	618	55	79	77	24	38	39	6.18				20
10	63.35	1400	1260	1309	497	626	85	74	95	15	28	33	6.45	141	4.2	270	20
11	63.45	1400	1510	1444	470	710	91	76	103	18	31	35	6.33				20
12	63.60	1400	1340	1337	490	657	107	67	114	10	28	31	6.70	144	4.2	280	25
13	63.70	1400	1640	1527	456	748	107	75	123	16	39	32	6.47				30
14	63.95	1400	1480	1406	465	688	105	69	114	13	34	35	6.62				30
15	63.70	1400	990	1537	649	643	87	68	89	14	38	36	6.56	144	3.6	278	25
16	64.10	1400	1400	1502	494	691	103	61	115	15	39	31	6.49				20
17	64.10	1400	1500	1601	510	765	113	67	116	16	48	40	6.55	142	3.5	278	20
18	64.15	1600	1380	938	365	415	96	42	91	5	26	14	6.89				20
19	64.50	1400	1125	1433	547	615	85	55	93	15	40	29	6.45				20
20	64.45	1500	1900	1716	434	825	129	69	150	16	44	40	6.55	142	3.7		20
21	64.30	1500	1760	1158	324	570	104	50	111	9	34	24	6.69				20
22	64.50	1500	1530	1297	465	697	116	61	123	14	46	33	6.65	141	3.2		20
23	64.35	1550	1710	1546	447	693	126	60	130	12	42	32	6.76				20
24	64.30	1550	1860	1597	459	711	118	63	128	15	38	28	6.66				20
25	64.10	1550	1370	1443	407	631	111	59	106	9	37	31	6.81	150	3.7	289	20
26	64.10	1550	1270	1485	571	725	122	64	125	13	56	40	6.63	146	3.4	281	20
27	64.10	1550	1670	1745	479	800	120	64	130	14	71	45	6.66	142	3.5	282	20
28	64.20	1600	2110	1332	339	716	140	67	146	7	36	33	6.96	144	3.5	281	20
29	64.00	1600	1580	1581	425	671	113	63	114	12	41	40	6.79	150	3.6	293	20
30	64.05	1500	1530	1590	501	752	136	63	130	11	45	38	6.86	147	3.7	284	20
31	64.05	1550	1390	1614	515	799	156	67	154	8	37	36	6.87	149	3.6	290	20

* Titratable acidity
** Desoxycorticosterone acetate in oil.

hypotonic to plasma in Subject J.F. was accompanied by a decreased excretion of osmotically active urinary components other than sodium and chloride. This initial water diuresis on day 13, with a urine low in osmolality and osmotic content, recurred on day 16. Subsequently, on day 21 when the natriuresis of the "escape" was maximal, urinary osmolality remained low, but in contrast to the initial water diuresis the total osmotic content rose to the high levels characteristic of osmotic diuresis. Although daily determinations of serum osmolality were not made during the cyclical

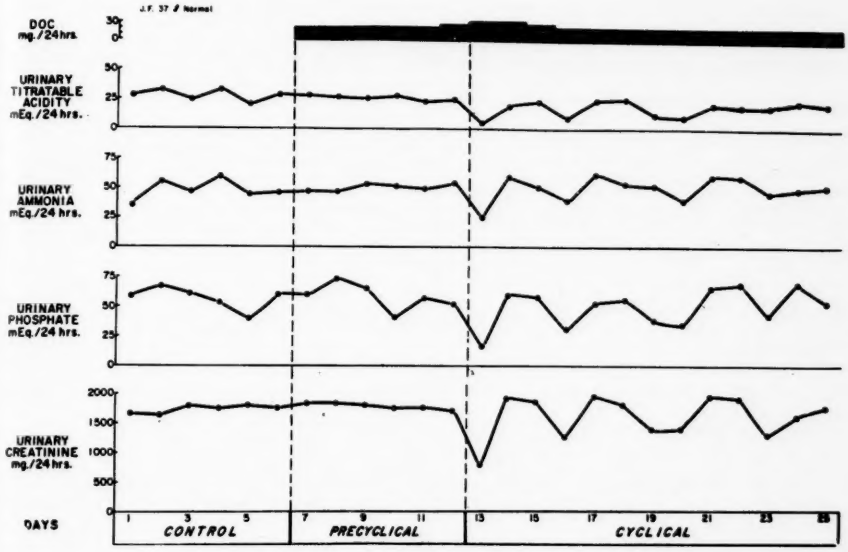

FIG. 2. The cyclical response of urinary creatinine, phosphate and acidification patterns induced by DOC in Subject J.F.

period in Subject J.F., it is evident that each rise in urinary sodium was preceded by the elaboration of a dilute urine of increased volume with low osmotic content, representing a net loss of nonosmotically obligated (free) water which coincided with the cyclical depressions in urinary creatinine, potassium, ammonia, hydrogen ion and phosphate.

Similar results were observed in Subject T.C. On day 18, coincident with the initial decrease in urinary creatinine, the osmolality fell from 510 mOsm. to 365 mOsm. per Kg. of water, with a decrease in osmotic content from 765 mOsm. per twenty-four hours to 415 mOsm. (Table 1). Although urinary sodium and chloride fell slightly, this could account for only about 12 per cent of the fall in osmotic excretion, and therefore the decrease in

osmolality and osmotic content again was achieved chiefly by the retention of solute other than sodium and chloride. A similar pattern occurred on day 21. Figure 5 illustrates the 12-hour patterns of free water clearance (C_{H_2O}) and osmotic clearance (C_{Osm}) in Subject T.C. from day 25 to the time of "escape" on day 31. Sodium excretion together with C_{Osm} progressively increased from day 28 to day 31, with increased losses occurring principally

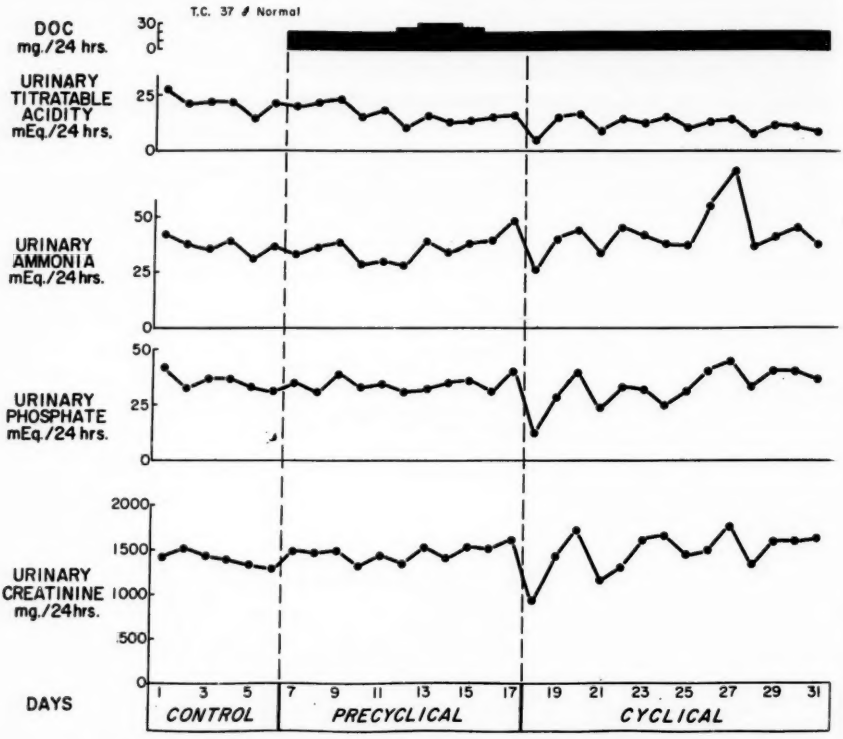

FIG. 3. The cyclical response of urinary creatinine, phosphate and acidification patterns induced by DOC in Subject T.C.

at night. In fact, on day 31 when natriuresis was maximal, 72 per cent of the sodium was excreted between 7:00 p.m. and 7:00 a.m. On the other hand, C_{H_2O} rose to a daytime maximum of +0.24 ml. per minute on day 28, and subsequently cycled every twelve hours with peaks of elevation during the day and depression at night. During the night of day 31 when maximal sodium excretion occurred, C_{H_2O} had declined to −1.36 ml. per minute. Thus, each nocturnal rise in sodium excretion and C_{Osm} was preceded by a relative rise in C_{H_2O} during the previous day.

Effect on serum sodium and potassium levels and osmolality

The average concentration of serum sodium and total solutes was virtually unchanged throughout the control and precyclical periods (Tables 1 and 2), except for slight depressions in serum sodium concentration and osmolality in both subjects during the precyclical period. However, in Subject T.C., at a time when increases in C_{H_2O} were observed, more fre-

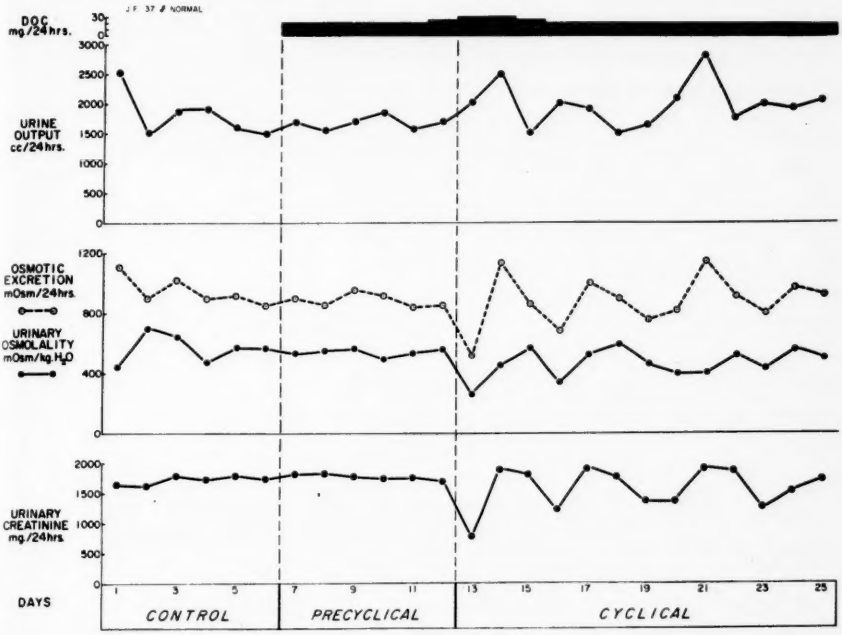

FIG. 4. The cyclical response of urinary output, osmolality and total solute excretion induced by DOC in Subject J.F.

quent serum determinations revealed rather marked fluctuations over a 12-hour period in both serum sodium concentration and osmolality (Fig. 5). Sodium concentrations ranged from 142 mEq. to 150 mEq. per liter during the one-week period immediately prior to maximal urinary sodium loss. Serum osmolality during this period fluctuated between 280 mOsm. and 293 mOsm. per Kg. of water, paralleling the sodium peaks.

Effect on steroid excretion

Although fluctuations in 17-hydroxycorticoid excretion were fairly marked during the adaptation to DOC in Subject J.F., no significant differences were observed from similar variations during the control period.

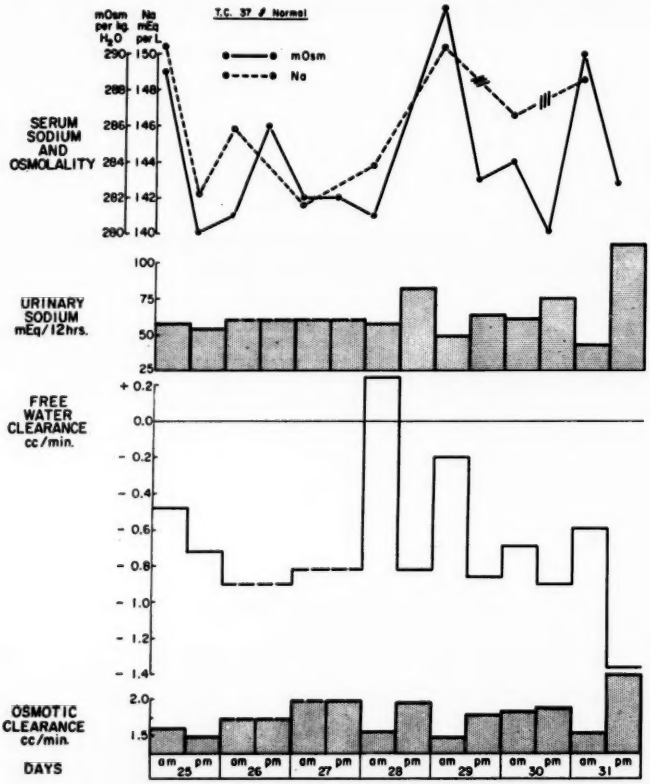

FIG. 5. Changes in free water clearance and serum sodium level and osmolality induced by DOC in Subject T. C. Reversal of the diurnal variation in sodium excretion is evident from days 28 to 31, when the "escape" from sodium retention occurred. The broken horizontal lines on days 26 and 27 represent average 12-hour values for single 24-hour urine specimens. All other urinary values are for 12-hour specimens; "am" = 7 a.m. to 7 p.m.; "pm" = 7 p.m. to 7 a.m.

DISCUSSION

Although the mechanisms responsible for the eventual increase in urinary sodium excretion in the presence of a powerful hormonal stimulus to salt retention are unknown, the role of increased extracellular fluid volume has assumed greater importance in eliciting adaptive changes in such important variables as intrarenal hemodynamics, specific tubular reabsorptive mechanisms, extracellular tonicity, and neurohumoral regulation. Despite conflicting results obtained in the study of such variables, it is evident that a dominant characteristic of the normal subject is a response to various stresses in a cyclical fashion.

Two patterns of renal excretory cycles are apparent in the normal subject which are generally absent or inverted in the edematous patient with cirrhosis, nephrosis or congestive heart failure—the well-known diurnal rhythm with its peak solute and water excretion during the day, and the less well-recognized tides of water and electrolyte excretion which occur in peaks every four to six days (27–30). A feature of both excretory rhythms has been the parallelism of sodium, potassium, chloride and water throughout the various cycles. In the present study both subjects showed a striking alteration of the normal five-day cycle (established in a subsequent study in subjects receiving an identical diet). Although the parallelism persisted, heightened peaks of excretion and retention of sodium and water occurred every one to three days, rather than the usual four to six days. In addition, Subject T.C. exhibited a reversal of his normal diurnal pattern, with a maximal nocturnal natriuresis at the time of "escape."

The initial increase in free water clearance in Subjects J.F. and T.C. at the beginning of the cyclical period was achieved mainly by a decreased excretion of solute other than sodium and chloride. The marked cyclical decrease in creatinine at this time may be attributed either to a decrease in the glomerular filtration rate (GFR), to an inhibition of that portion of creatinine believed to be secreted by the renal tubules (31), or to a combination of these factors. Moderate reduction of the GFR in man and dog is regularly accompanied by a decrease in urinary flow and a fall in sodium excretion (32, 33). The finding of an increased urinary flow and an unchanged sodium excretion in Subject J.F. and only a very slight decrease in these parameters in Subject T.C. suggests that the large simultaneous decreases in creatinine excretion in both subjects were the result of an inhibition of tubular secretion or an inhibition out of proportion to any fall in GFR. Moreover, the simultaneous depression of such diverse tubular processes as hydrogen-ion secretion, urinary concentration, potassium secretion, ammonia production and probably creatinine secretion suggests such a major and widespread alteration as decreased effective blood supply to these renal tubular areas. The possibility of such a decrease in blood supply is supported by recent studies which have shown a pronounced fall in renal plasma flow (C_{PAH}) with an attendant rise in the filtration fraction prior to the period of maximal sodium loss (34).

The significance of the cyclical depression of the various urinary components and the accompanying rise in free water clearance is unknown. The hypernatremia and hyperosmolality accompanying this elevation in water excretion may facilitate the excretion of sodium by an increase in the filtered load. The rise in free water clearance accompanying the acute expansion of extracellular fluid volume with isosmotic saline has been shown to be significantly dependent upon posture (35, 36). This observa-

tion suggests that in the present study the alternating loss of water and sodium may represent more complex postural adaptations of the erect human which have not been observed during prolonged administration of DOC to dogs (37). In the normal human subject, therefore, the ultimate pattern of natriuresis in response to DOC-induced sodium retention may represent both postural and volume-induced changes in the mechanisms for the renal handling of sodium.

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CLINICAL ASSESSMENT OF BODY HAIR GROWTH IN WOMEN

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ABSTRACT

A method was developed for the semiquantitative assessment of body hair growth, and suitable for use in the study of clinical problems associated with hirsuties in women. Five gradings based on densities and areas involved, were determined for each of 11 sites. Findings from application of the method to a control group of 430 women (ages, 15–74 years) are reported. Hair tended to increase on the face and disappear from all other sites with advancing years. In younger age groups a significant amount of hair was found on the forearm and leg in most subjects, but a zero grading was much the commonest finding at all other sites. It is suggested that 2 factors may be involved: one of protective nature with main expression on the forearm and leg, and the other related to hormone levels or sensitivity, with clearest expression elsewhere. An "hormonal" score obtained by adding the gradings obtained from 9 of the 11 sites (excluding the forearm and leg) is being employed in clinical studies.

CLINICAL problems related to hirsuties in women have usually been approached as if there were a clear dividing line between hirsuties and the normal state. Body hair growth appears, however, to be a graded characteristic (1). A method for assessing hair growth is needed which will be quantitative and, at the same time, suitable for clinical use. A number of studies of hair growth in men and women have been reported (2–9) since the pioneer work of Danforth and Trotter in 1922 (10). The problem of assessment resolves itself into a selection of suitable sites for study, and the choice of a method for grading hair growth at any given site.

The various sites are not involved consistently in the female; for example, the face and chest may be affected in one, and the lower abdomen and thighs in another. It is clearly desirable, therefore, to study a number of sites. The idea of grading hair growth at any site according to the density and the area involved was introduced by Dupertuis, Atkinson and Elftman (3), and developed by Garn (5). The involvement of any site with increasing hair growth tends to follow a definite pattern, so that it is not difficult

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to select a number of gradings at each site. Hair growth is scored according to the sum of the gradings obtained. This method is comparatively precise, since the spread of scoring is considerable. It is considered the best method devised so far for clinical use. More objective methods such as weighing or counting hairs are clinically impractical, especially if a number of sites are to be studied.

The nature of the scoring in the population at large must be known for any method before it can be of use in clinical studies. The method adopted in this department has therefore been applied to a control series of 430 women and the findings form the basis of the present communication.

MATERIAL AND METHODS

Adequate co-operation in an investigation of this nature was unlikely to be forthcoming from any group of subjects outside a hospital. It was therefore decided to study a consecutive series of women attending a general medical out-patient clinic. It was thought that the series would be sufficiently representative of the population at large for the purposes of this study, provided care was taken to exclude patients suffering from diseases or symptom complexes which might be associated with disturbances of hair growth (including diseases of the anterior pituitary, adrenal cortex and ovary, hypothyroidism, generalized skin diseases, menstrual disturbances and infertility). Only a few such cases were encountered. No patients came complaining of excessive hair growth, but a few had mild hirsuties. This hirsuties was considered to be constitutional in origin, and further study was not considered necessary on clinical grounds nor for the furtherance of this particular investigation.

Four hundred and thirty women were studied. Their ages ranged from 15 to 74 years.

The method of assessment was based upon that of Garn (5). Eleven sites were studied —lip, chin, chest, upper back, sacro-iliac region, upper and lower abdomen, arm and back of forearm, thigh and leg (Figs. 1 and 2). Only terminal hair growth was considered, and 5 grades were determined for each site, zero grading in each instance being an absence of terminal hair. Definitions of the gradings are shown in Table 1. The gradings for one of these sites are illustrated in Figure 3. Most of the women could be classified quite readily by this system. In some borderline cases, a subjective impression of density determined the final grading.

RESULTS AND CONCLUSIONS

The data on the 430 women, analyzed by age decades, are listed in Table 2. Study of the findings led to certain conclusions.

Age range for comparison of hair growth

It can be seen that after the age period 45–54, hair tended to increase on the face and to disappear from all other sites. A similar effect of age has been observed by other workers (2, 4, 9, 11). Adolescence is known to be a period of increasing hair growth. The age range in which the hair growth of subjects can be compared, therefore, cannot be precisely defined, but it is around 20–40 years.

TABLE 1. DEFINITION OF HAIR GRADINGS AT EACH OF 11 SITES
(Grade 0 at all sites indicates absence of terminal hair.)

Site	Grade	Definition
1. Upper Lip	1	A few hairs at outer margin.
	2	A small moustache at outer margin.
	3	A moustache extending halfway from outer margin.
	4	A moustache extending to mid-line.
2. Chin	1	A few scattered hairs.
	2	Scattered hairs with small concentrations.
	3 & 4	Complete cover, light and heavy.
3. Chest	1	Circumareolar hairs.
	2	With mid-line hair in addition.
	3	Fusion of these areas, with three-quarter cover.
	4	Complete cover.
4. Upper back	1	A few scattered hairs.
	2	Rather more, still scattered.
	3 & 4	Complete cover, light and heavy.
5. Lower back	1	A sacral tuft of hair.
	2	With some lateral extension.
	3	Three-quarter cover.
	4	Complete cover.
6. Upper abdomen	1	A few mid-line hairs.
	2	Rather more, still mid-line.
	3 & 4	Half and full cover.
7. Lower abdomen	1	A few mid-line hairs.
	2	A mid-line streak of hair.
	3	A mid-line band of hair.
	4	An inverted V-shaped growth.
8. Arm	1	Sparse growth affecting not more than a quarter of the limb surface.
	2	More than this; cover still incomplete.
	3 & 4	Complete cover, light and heavy.
9. Forearm	1, 2, 3, 4	Complete cover of dorsal surface; 2 grades of light and 2 of heavy growth.
10. Thigh	1, 2, 3, 4	As for arm.
11. Leg	1, 2, 3, 4	As for arm.

Factors underlying body hair growth

In the 20–40 age group, it was observed that in most of the women a significant growth of hair was present on the forearm and leg, though a zero grading was much the commonest elsewhere. This feature was submitted to further study, attention being directed to the 161 women between the ages of 18 and 38. Two scores were obtained for each subject, one being the sum of the gradings for the forearm and leg, and the other the sum of the gradings for all other sites. The percentage incidences of the various scores obtained for the forearm and leg were calculated, as were those for all

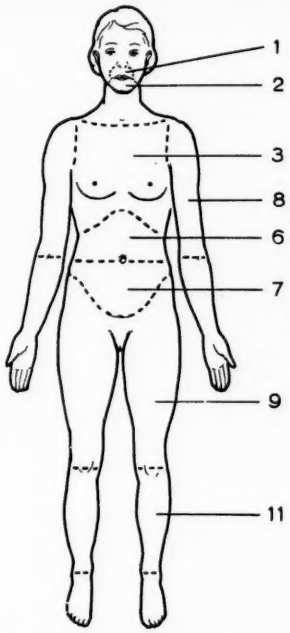

FIG. 1. Demarcation of sites—anterior view.

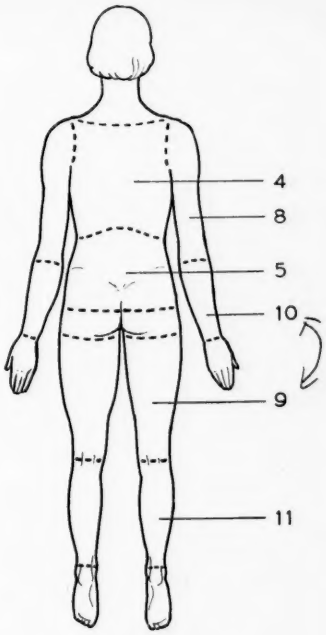

FIG. 2. Demarcation of sites—posterior view.

other sites. The findings are contrasted graphically in Figures 4 and 5. The 2 curves differ greatly and it is possible that 2 factors underlie body hair growth. One type of growth, with main expression on the forearm and leg, may be sexually indifferent and protective in nature. The other, with clearest expression elsewhere, may be sexually determined and related to blood hormone levels or to sensitivity of hair follicles to circulating hormone. The action of the 2 factors must overlap. For reference purposes the sum of the gradings on the forearm and leg has been termed the "indifferent" score, and the sum of the gradings from all other sites, the "hormonal" score. Figure 6 shows corresponding "indifferent" and "hormonal" scores for each of the 161 subjects; r for the group was 0.48, and this showed a significant correlation ($P < 0.001$).

Assessment of hirsuties

The score for "hormonal" sites alone can now be employed in the study of clinical problems concerned with hirsuties in women. Examination of

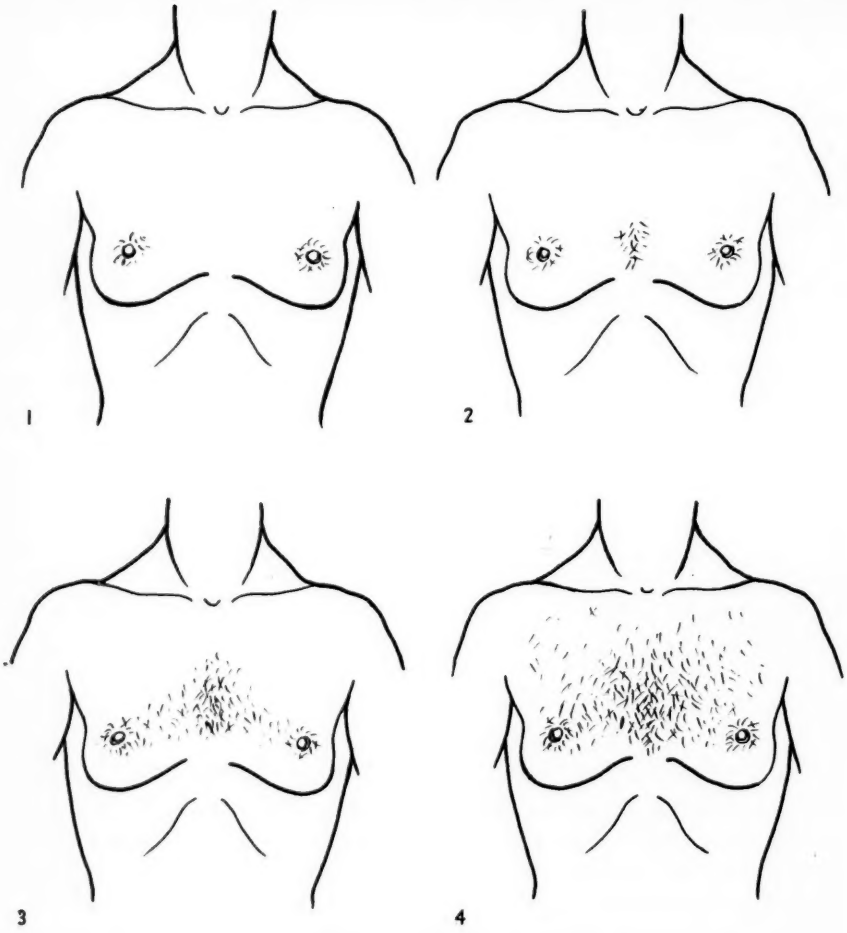

FIG. 3. Hair growth gradings—front of chest.

findings in the present series of 161 women aged 18 to 38 permitted assessment of the significance of such scores found in clinical practice. An "hormonal" score above 5 was found in 9.9 per cent (16 women), and above 7 in 4.3 per cent (7 women), but scores above 10 were found in only 1.2 per cent (2 women).

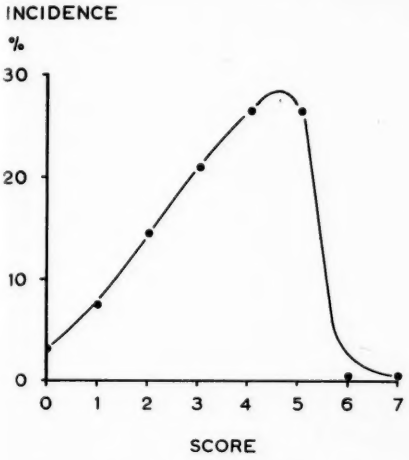

FIG. 4. Percentage incidence of the sums of the gradings for the forearm and leg ("indifferent" scores) in 161 patients aged 18–38.

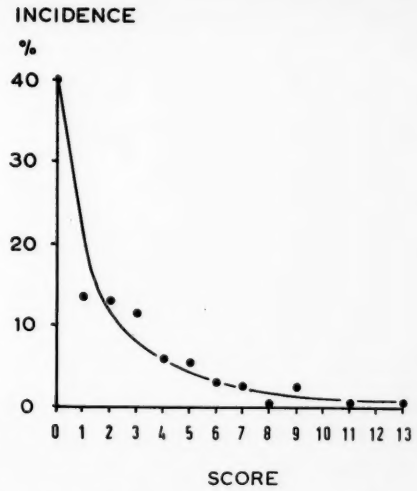

FIG. 5. Percentage incidence of the sums of the gradings for the lip, chin, front of the chest, upper and lower back, upper and lower abdomen, arms and thighs ("hormonal" scores) in 161 patients aged 18–38.

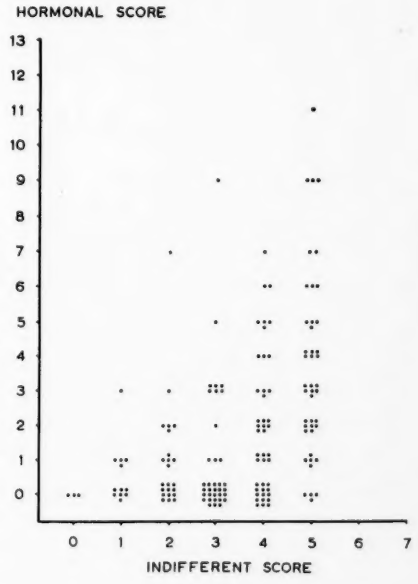

FIG. 6. Correspondence of "indifferent" and "hormonal" scores for each of the 161 patients aged 18–38.

TABLE 2. PERCENTAGE FREQUENCY DISTRIBUTION OF HAIR-GROWTH GRADINGS AT 11 SITES, BY AGE DECADES

Age decade	No. of cases	Site	Gradings				
			0	1	2	3	4
15–24	75	Lip	71	19	10		
		Chin	98	2			
		Chest	93	6	1		
		Upper back	99	1			
		Lower back	86	13	1		
		Upper abdomen	100				
		Lower abdomen	72	18	7	2	1
		Arm	74	21	5		
		Thigh	57	27	13	3	
		Forearm	9	24	62	5	
		Leg	5	11	35	46	3
25–34	92	Lip	61	25	11	3	
		Chin	89	5	6		
		Chest	86	12	2		
		Upper back	100				
		Lower back	88	12			
		Upper abdomen	100				
		Lower abdomen	79	12	3	5	1
		Arm	77	19	4		
		Thigh	68	14	15	3	
		Forearm	14	46	36	4	
		Leg	3	4	36	53	4
35–44	90	Lip	47	28	19	5	1
		Chin	84	9	7		
		Chest	76	18	6		
		Upper back	100				
		Lower back	86	14			
		Upper abdomen	99	1			
		Lower abdomen	77	20	2	1	
		Arm	93	7			
		Thigh	71	11	16	2	
		Forearm	40	40	20		
		Leg	9	17	46	27	1
45–54	83	Lip	40	32	15	10	3
		Chin	85	6	6	3	
		Chest	93	7			
		Upper back	100				
		Lower back	99	1			
		Upper abdomen	100				
		Lower abdomen	91	4	2	3	
		Arm	98	1	1		
		Thigh	75	13	8	2	
		Forearm	57	27	14	2	
		Leg	25	23	46	6	
55–64	50	Lip	36	24	20	16	4
		Chin	66	20	10	4	
		Chest	100				
		Upper back	100				
		Lower back	98	2			
		Upper abdomen	100				
		Lower abdomen	94	4	2		
		Arm	100				
		Thigh	86	6	8		
		Forearm	84	6	8	2	
		Leg	62	16	22		

Table 2—(continued)

Age decade	No. of cases	Site	Gradings				
			0	1	2	3	4
65–74	50	Lip	46	12	16	18	8
		Chin	66	20	4	8	2
		Chest	100				
		Upper back	100				
		Lower back	100				
		Upper abdomen	100				
		Lower abdomen	100				
		Arm	100				
		Thigh	96	4			
		Forearm	84	16			
		Leg	86	12	2		

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RESIN UPTAKE OF RADIOTHYROXINE FROM SERUM IN THYROID DISEASE AND IN PREGNANCY*†

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ABSTRACT

Further studies on the *in vitro* resin uptake of thyroxine-I^{131} from serum in thyroid disease and in pregnancy are described. Aliquots of serum containing thyroxine-I^{131} were equilibrated in test tubes with 1.0-ml. volumes of the anion exchange resin, Amberlite IRA-400, prepared with tris-maleate buffer, pH 5.2. The fraction of radioactivity remaining on the resin, expressed as a percentage of the normal control value, was significantly higher in hyperthyroidism and lower in pregnancy and in hypothyroidism than in the normal control subjects. These data suggest that the resin procedure provides an *in vitro* method for evaluating thyroid function and distinguishing between the nonpregnant and the pregnant state.

THE technique for *in vitro* determination of the resin uptake of I^{131}-labeled hormones was first introduced in order to demonstrate differences in thyroxine binding by the serum of pregnant women (1). Thereafter, the resin was used to assess other conditions in which alterations in hormone binding were evident, such as the binding of insulin-I^{131} by serum from insulin-treated diabetic patients (2). Particularly striking were the differences in the resin uptake of thyroxine-I^{131} (T_4-I^{131}) by the serum of patients with hyperthyroidism and hypothyroidism contrasted with the uptake in normal subjects (3).

This report summarizes the results of resin determinations on the sera of normal controls, pregnant women and patients with hyperthyroidism and hypothyroidism. The data indicate that the resin uptake of T_4-I^{131} continues to be a useful means for distinguishing between normal and abnormal thyroid function and for recognizing pregnancy.

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MATERIALS AND METHODS

The method described in earlier publications (1–3) differs from that outlined here by virtue of a variation in the buffer or the I^{131}-labeled hormone used.

Sera were obtained from healthy volunteers, patients with unequivocal hyperthyroidism or hypothyroidism, and women with uncomplicated pregnancies. The sera were stored frozen, and frequently the same serum underwent repeated freezing and thawing before use. Sera kept stored at 4° C. were not always consistent in behavior; in repeated determinations on the same serum, the resin uptake values tended to increase. Plasma could not be used because of the clumping and strand formation which took place within the resin beads.

Amberlite[1] IRA-400, a strongly basic anion exchange resin, was washed by decantation with several volumes of water (double-distilled in glass) until the supernatant was free from discoloration and particulate matter. Tris-maleate buffer[2] (0.2*M*), pH 5.0 to 5.2, was added in a volume ratio of 8 to 1 of the wet resin. The mixture was stirred for two hours at 3° C.; then the buffer was decanted and replaced by fresh tris-maleate solution. The resin was allowed to equilibrate in the cold until the pH of the slurry remained constant. The tris-maleate buffer was discarded and the resin washed several times with distilled water. The resin was poured as a slurry into a sintered glass funnel (medium) and the excess water was permitted to drain by gravity. Some of the wet resin was transferred to a glass plate where a portion of it was introduced into a 10-ml. syringe with the aid of a spatula blade (Fig. 1).

The end of the syringe barrel had been removed at the 1-ml. graduation in order to facilitate the delivery of the resin. Uniform packing was achieved and the excess water expressed by placing the open end of the syringe against the glass plate and pressing the plunger down against the head of the resin. One-ml. volumes of the damp resin were then measured into graduated Pyrex test tubes (16 mm. × 100 mm.) by cutting the resin with the spatula blade as it was extruded from the syringe. Resin beads adhering to the sides of the glass tubes were washed down with a stream of distilled water. The individual tubes of resin were washed repeatedly with distilled water until the supernatants (removed by aspiration with a suction flask) were no longer turbid.

A 2-ml. aliquot of serum was equilibrated approximately thirty minutes with 0.01 to 0.03 ml. of a 1 per cent albumin solution containing approximately 0.05 μc. of I^{131}-labeled *l*-thyroxine[3] and 0.004 to 0.0005 μg. of stable thyroxine. Duplicate 1-ml. aliquots of the serum were added to the tubes containing the resin. The stoppered tubes, inclined at a 30° angle to the horizontal, were shaken at 60 oscillations per minute for one and a half hours at 3° C., after which the radioactivity within the tubes was measured in a well-type scintillation detector. The resins were washed three to four times by filling the tube completely with distilled water, permitting the resin beads to settle before aspirating the supernatant solution. After the final washing, sufficient distilled water was added to the tube to equal the starting volume and the radioactivity was measured again. The fraction of T_4-I^{131} bound by the resin was calculated by dividing the residual radioactivity by the initial radioactivity. The average of the duplicate values was used.

Since variations in the results occurred from time to time in all the groups, absolute limits for normal and abnormal could not be defined. By arbitrarily designating the maximal resin uptake of T_4-I^{131} of the control sera to represent 100 per cent, the other

[1] Rohm and Haas Co., Philadelphia 5, Pa. (Obtained in the chloride cycle.)

[2] Tris(hydroxymethyl)aminomethane-maleate (tris-maleate) buffer.

[3] Abbott Laboratories, Oak Ridge, Tenn.

results in any set of determinations may be converted to the corresponding percentile values. The distribution for both normal and abnormal sera may be obtained and finite limits described for the various groups. The range of normal values was established first

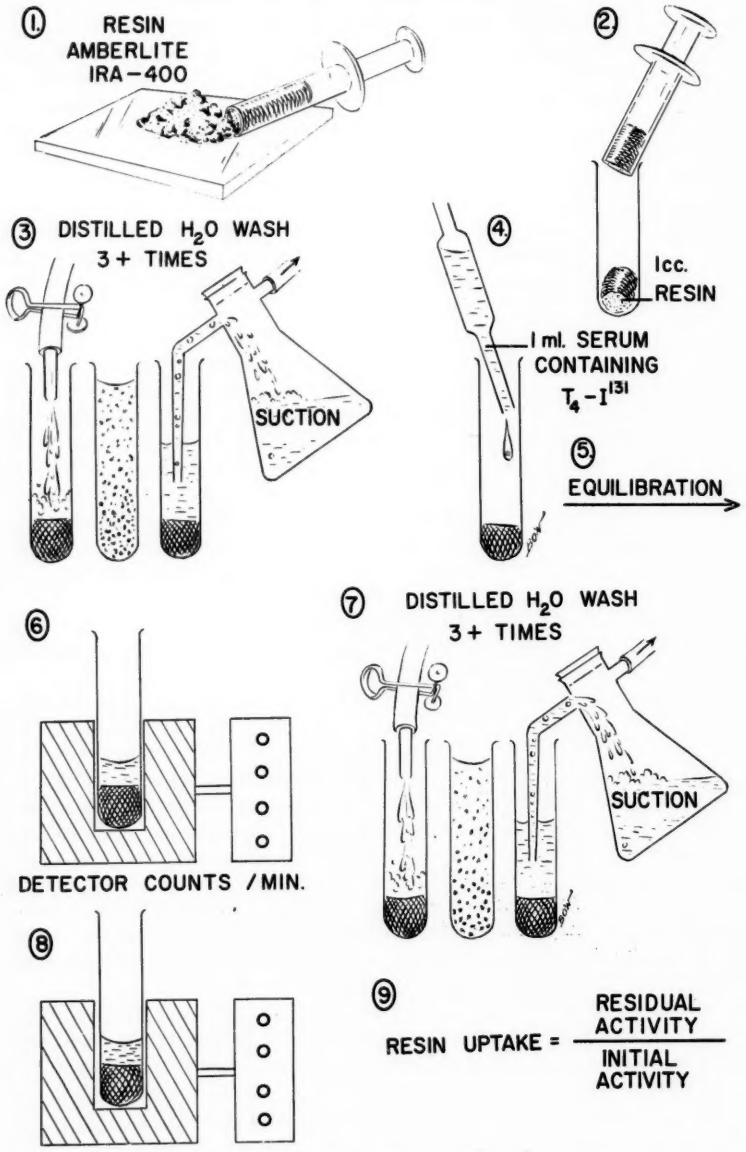

FIG. 1. *Resin Method.* The steps in the technique are outlined in numerical sequence.

by determining the resin uptake of T_4-I^{131} for serum from 40 normal subjects. Thereafter, the serum with the highest resin uptake, along with serum from at least 2 other normal subjects, was placed in each set of determinations. Additional sera from normal volunteers were constantly used in order to replenish the earlier supply.

If pooled normal serum were to be used as a reference standard, it would be necessary first to determine the relationship of the resin value for the pooled serum to the range of normal values in order to interpret properly the results for the abnormal sera.

The following calculations were used in the conversion of the resin uptake to percentage values:

$$\text{Normalized value } (\%) = \frac{\text{Resin uptake of } T_4\text{-}I^{131} \text{ from abnormal serum}}{\text{Maximal resin uptake of } T_4\text{-}I^{131} \text{ from normal serum}} \times 100.$$

RESULTS

Duplicate determinations

The mean difference between the duplicate values was 1.5 ± 1.0 (1 S.D.) and the duplicate variability for 95 per cent of the determinations was less than 3.0 per cent. Duplicate determinations of borderline values which did not agree within 3.0 per cent were repeated. Slight differences in the resin volumes between the duplicates appeared to have no effect upon the duplicate measurements.

Group values

The normalized results of the group determinations in normal, hyperthyroid, hypothyroid and pregnant subjects are represented in Figure 2.

Normal subjects. In 280 determinations carried out on 146 normal volunteers, the normalized mean resin uptake value was 93.7 ± 0.9 per cent (2 S.E.).

Hyperthyroidism. In 71 determinations on 57 previously untreated thyrotoxic patients, the normalized mean resin uptake value was 112.7 ± 4.6 per cent.

Hypothyroidism. In 74 determinations on 59 hypothyroid patients, the normalized mean resin uptake value was 81.6 ± 2.2 per cent.

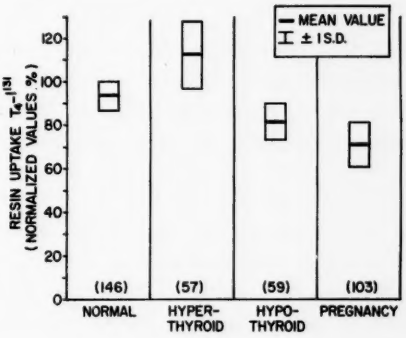

FIG. 2. Differences in resin uptake of T_4-I^{131} from serum in normal subjects, thyroid disease, and pregnancy. The figures at the bottom of each column indicate the number of subjects in each group.

Pregnancy. In 129 determinations on 103 women with uncomplicated pregnancies, the normalized mean resin uptake value was 71.8 ± 2.7 per cent.

All the group values were significantly different when compared with the values for the normal controls ($P = <0.001$).

Effect of pH

Figure 3 illustrates the effect of variations in pH upon the uptake of T_4-I^{131} by the resin. Enhancement of resin binding occurred with increasing

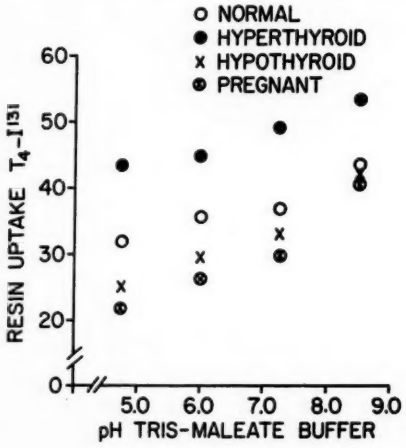

FIG. 3. Effect of pH upon the resin uptake of T_4-I^{131} from serum. The absolute fraction of T_4-I^{131} bound by the resin increased as the pH of the buffer was raised.

pH values. However, in spite of the augmented resin uptakes of radiothyroxine, the relative differences between the groups were either unchanged or narrowed. The results indicated that the pH range from 4.7 to 6.0 (with tris-maleate buffer) was optimal for achieving clear separation between the groups.

Effect of antithyroid compounds

Propylthiouracil added to serum *in vitro* had no measurable effect upon the resin uptake of T_4-I^{131}. However, as illustrated in Figure 4, the administration of propylthiouracil to patients with hyperthyroidism resulted in marked reductions in the resin uptake values.

Comment

The resin uptake values for the sera of some patients with a variety of acute and chronic illnesses were appreciably higher than those of normal subjects. As noted previously (4), thyrotoxicosis coincident with pregnancy prevented the usual reduction in resin values observed in pregnant women.

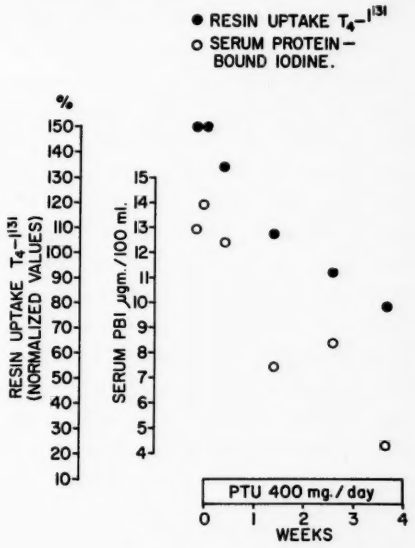

FIG. 4. Effect of propylthiouracil upon resin uptake of T_4-I^{131} from serum in a patient with hyperthyroidism.

DISCUSSION

In the original description of the resin technique, 1 ml. of serum containing the I^{131}-labeled hormone was equilibrated with 1 ml. of the anion exchange resin, Amberlite IRA-400, in the chloride or acetate cycle. Later, tris-maleate buffer was used in an effort to eliminate the variations in the results which occurred with T_4-I^{131} or triiodothyronine-I^{131}(T_3-I^{131}) (4). The tris-buffer succeeded in reducing the magnitude and the frequency of the variations, but not in abolishing them completely. Sporadic fluctuations caused the absolute value for each group to shift slightly without changing the relative differences between the groups. Difficulties arose, therefore, not in the clinical interpretation of the results, but in attempting to express normal and abnormal values in absolute terms. This has now been resolved by reporting the results as a percentage of the normal value.

The preparation of this report was purposely delayed in the hope that a specific resin, buffer, or I^{131}-labeled analogue of thyroxine would insure consistently uniform results. This hope has not materialized in association with use of the resin beads, but has been borne out with the development of resin sponge (5). The latter material yields consistently uniform results, entails a minimum of manipulation, requires no special preparation before use, and is stable at room temperature. For technical ease, simplicity of procedure, reproducibility of results, and for distinction between group values, the resin sponge is to be preferred over the resin beads. However, until the resin sponge becomes commercially available, the resin beads,

even with the limitations of the procedure, provide a means of evaluating thyroid function *in vitro*. This is particularly rewarding in circumstances where the conventional methods for assessing thyroid function cannot be applied. When hyperthyroidism occurs during pregnancy, the usual clinical and biochemical changes may be of no help in arriving at a correct diagnosis. The resin procedure appears to have resolved this problem. The failure to find a low resin uptake from the serum of a pregnant woman in the presence of an elevated protein-bound iodine level strongly suggests that hyperthyroidism may be present.

The overlap of values between the hyperthyroid and the normal group was not as great as that between the hypothyroid and the normal group. It is of interest that similar results have been obtained by others (6) using the erythrocyte uptake of T_3-I^{131} (7). This suggests that in hypothyroidism, the lack of clear delineation of a range of values may lie in the nature of the disease and may not necessarily be the fault of the procedure.

The substitution of T_3-I^{131} for T_4-I^{131} resulted in higher values without changing the relative differences between the groups or improving the discrimination between them. Sterling and Tabach recently confirmed this observation; they used the technique outlined here, but substituted formate for tris-buffer (8).

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HYPERTHYROIDISM AND DECREASED THYROXINE BINDING BY SERUM PROTEINS*

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ABSTRACT

In 2 sisters, hyperthyroidism was associated with normal levels of serum protein-bound iodine (PBI). A decrease in thyroxine (T_4) binding by serum alpha-globulin (TBG) and pre-albumin (TBPA) was demonstrated in both cases by paper electrophoresis in ammonium carbonate buffer. TBG capacities were 0.08 and 0.11 μg. of T_4 per ml., and the serum PBI levels were 5.1 and 6.6 μg. per 100 ml., respectively. TBG capacity among normal subjects ranged from 0.13 to 0.25 μg. of T_4 per ml. The reduction in the TBG level is offered as the most likely explanation for the normal concentration of serum PBI in these patients. In one of the cases, repeated studies following radioiodine therapy revealed an increase in TBG and TBPA levels to low-normal and a fall in PBI concentration to an average value of 4.0 μg. per 100 ml. Chromatographic analysis of the serum I^{131} compounds showed a normal ratio of T_4 to T_3. An incidental finding in both patients was the presence in the serum of an iodinated protein comprising nearly one-sixth of the protein-bound I^{131}. A genetically determined defect is postulated to account for the occurrence in these sisters of hyperthyroidism and decreased serum thyroxine binding.

THE level of serum protein-bound iodine (PBI) is commonly taken to represent the concentration of thyroid hormone in the blood. In the absence of contamination by nonhormonal iodine, therefore, the PBI level should be, and usually is, a reliable indicator of thyroid status (1). There are, however, exceptions to this rule, and these deserve attempts at explanation.

Thyroxine, which comprises the largest portion of the protein-bound iodine, is associated with 3 protein components when serum is analyzed by paper electrophoresis. In order of decreasing affinity for thyroxine, these proteins are:

1. Thyroxine-binding alpha-globulin (TBG) (2, 3).
2. Thyroxine-binding pre-albumin (TBPA) (4).

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3. Serum albumin (2, 3).

Anomalous PBI levels in certain conditions not associated with diseases of the thyroid have been explained by observed alterations in the binding capacity of TBG. In each of the following examples, parallel changes in the PBI level and in serum thyroxine-binding capacity tended to maintain normal blood concentration of diffusible (unbound) thyroxine, which is presumably the metabolically active fraction.

Increases both in serum PBI concentration and in the binding capacity of TBG occur in normal pregnancy (5, 6) and during estrogen administration (7). In such cases the change in TBG capacity is believed to be primary to the alteration in PBI (3). Beierwaltes and Robbins (8) reported an unexplained elevation in PBI and in TBG capacity in 2 apparently normal members of a family. Conversely, a decrease both in the PBI level and in TBG capacity has been found in some patients with nephrosis (3) and in euthyroid subjects treated with methyltestosterone (9). Apparent absence of TBG in association with normal binding by pre-albumin has been reported in 1 euthyroid male whose serum PBI level averaged 3.0 μg. per 100 ml. (10). Ingbar and Freinkel recently studied a similar case (11).

In all of the studies reported to date, TBG capacity in hyperthyroidism has been found to be normal (3, 11, 12). Ingbar and Freinkel noted a decrease in the binding capacity of TBPA in thyrotoxicosis as well as in a variety of other acute illnesses not directly involving the thyroid (11). However, the physiologic importance of TBPA is still in doubt. The level of TBPA has not been correlated with the level of PBI, and recently evidence has been presented to indicate that TBPA may not bind thyroxine at physiologic pH (13, 14).

It seemed logical, therefore, to search for alterations of thyroxine binding in the serum of hyperthyroid patients who exhibit unexpectedly low PBI values. The purpose of this paper is to report the findings in 2 sisters with hyperthyroidism in whom serum PBI levels were within normal limits. A reduction in TBG capacity was found in both cases and is proposed as an explanation for the "normal" PBI values. This is believed to be the first reported example of such a phenomenon.

METHODS

Determination of serum thyroxine-binding capacity was carried out by a modification of the method of Robbins (15). I^{131}-labeled sodium L-thyroxine was obtained from Abbott Laboratories and assayed for radiochemical purity by paper chromatography in n-butanol-dioxane-2*N* ammonia (40:10:50). Only those samples which were more than 90 per cent pure were used. A 2–3 μc. aliquot (0.05 to 0.15 μg.) of I^{131}-thyroxine was added to each ml. of serum. Aliquots were enriched with known amounts of stable sodium L-

thyroxine, dissolved in 1 per cent human albumin and physiologic saline to minimize adsorption of thyroxine to vessel surfaces. The amount of albumin added in this step was less than 1 per cent of the albumin present in the serum. After equilibration of the thyroxine-serum mixtures overnight at 4° C., the samples were subjected to reverse-flow paper electrophoresis at room temperature for sixteen to twenty hours in 0.1 *M* ammonium carbonate buffer which had been adjusted to pH 8.4 by bubbling carbon dioxide through the solution. The paper strips were dried, scanned for radioactivity in a strip counter, and placed in contact with x-ray film for one or two days so that areas of radioactivity could be located exactly. Corresponding sections of the paper strips were cut out and counted in a well-type scintillation counter. Duplicate determinations were run on each specimen of serum at each of two concentrations of added stable thyroxine—1.10 and 4.50 μg. of thyroxine per ml. of serum. Final calculation of binding capacity for prealbumin was based only on the results at the higher thyroxine level; the TBG value was obtained by averaging the results at both concentrations. The total thyroxine concentration was calculated from the endogenous PBI level determined by chemical analysis and assumed to represent only thyroxine, the specific activity of the added I^{131}-L-thyroxine, and the known amount of added stable thyroxine. In each determination of binding capacity, duplicate results varied by less than ±5 per cent.

CASE REPORTS

A. H. (propositus)

A 40-year-old married, white female complained of nervousness, irritability and difficulty in swallowing for approximately four months. She had sustained a 5-pound weight loss in spite of a normal food intake. Increased sensitivity to heat and profuse sweating were mentioned. She complained of some shortness of breath and rapid heart action after climbing a single flight of stairs. The past history was noncontributory. The menarche had occurred at age 15, and menstrual cycles were twenty-six days apart. The patient had never been pregnant. At the time of her initial visit she was taking no medication of any kind.

Physical examination revealed a slim, apprehensive woman with a normal blood pressure. The pulse was 120 and regular. The skin was warm and moist. A stare and lid-lag were present but there was no proptosis. The thyroid gland was diffusely enlarged to about twice normal size and felt boggy; a bruit was audible. There was a fine tremor of the hands. Ankle edema was 1-plus. The pelvic organs were normal, as were the other physical findings.

Urinalysis showed no albumin on two occasions. The blood hemoglobin level and leukocyte count were normal. The concentration of total serum protein was 6.6 gm. per 100 ml., and the electrophoretic pattern showed a slight reduction in the albumin fraction with an elevation in the alpha-globulin fraction. An electrocardiogram and a chest roentgenogram were normal. Urinary excretion of 17-ketosteroids was 10.1 mg. per day (normal, 6–18 mg.). The results of thyroid function studies are listed in Table 1. A scintiscan of the thyroid showed a diffuse pattern of activity.

The patient was treated with 5 mc. of I^{131} on May 14, 1960. Improvement in symptoms began about three weeks following treatment, and continued for four months. In October 1960, five months after I^{131} therapy, she had gained 9 pounds; she was asymptomatic and considered to be euthyroid by all clinical criteria. No symptoms of hypothyroidism developed, and one year after I^{131} therapy she is still euthyroid.

TABLE 1. LABORATORY FINDINGS IN PATIENT A.H.

Test	Reference to method	Normal range	Year 1960									Year 1961	
			Apr.	May		June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.
Serum PBI	(16)	4.0–7.4 μg./100 ml./	5.8	5.1*	5.8	4.6	6.6	2.4	3.9	3.4		4.1	4.2	4.1
Serum BEI	(17)	3.4–6.8 μg./100 ml.							2.5					
BMR		−15 to +15%		+17										+11
Serum cholesterol		125–325 mg./100 ml.		136									240	
I^{131} Thyroidal uptake (24-hour)		15–40% of dose		65			31						38	
Serum PBI^{131} (48-hour)		<0.4% of dose per liter		0.7									0.9	
Erythrocyte I^{131}-T_3 uptake	(18)	11–18% per 2 hrs.		27			15	13		18		22	19	16
Plasma I^{131}-thyroxine, half-time	(19)	5.9–7.3 days										5.5		
Extrathyroidal organic I pool	(19)	441–655 μg.										376		
Thyroxine degradation rate	(19)	46–68 μg./24 hrs.										47		
Serum thyroxine-binding capacity:														
TBG		0.13–0.25 μg. T_4/ml.		.08	.09	.12	.14	.11	.13			.13		.13
TBPA		1.30–2.70 μg. T_4/ml.		1.22	1.10	1.60	2.08	1.62	1.82			1.80		1.57

* A dose of 5 mc. of I^{131} was given May 14.

W. R. (sister)

This 37-year-old woman had undergone a subtotal thyroidectomy five years previously for a diffuse toxic goiter with a basal metabolic rate (BMR) of +53 per cent. The result was considered satisfactory; the symptoms disappeared and the BMR returned to normal. (Unfortunately, the serum PBI level was not determined at that time.) Approximately four years later she began to notice a return of symptoms of thyrotoxicosis—nervousness, weight loss, frequent bowel movements, and an enlarged neck.

On examination she was tense and tremulous, and her skin was warm. The pulse rate was 90 and regular. There was a staring gaze but no exophthalmos. A asymmetrically enlarged thyroid was easily felt beneath the thyroidectomy scar.

Laboratory findings at this time included a serum PBI level of 6.6 μg. per 100 ml. and an erythrocyte I^{13}-triiodothyronine(T_3) uptake of 23 per cent (normal, 11–18 per cent). The serum protein level was 6.4 gm. per 100 ml.; the albumin fraction was slightly low and both alpha- and beta-globulin fractions were slightly elevated.

Because the patient was clearly thyrotoxic by clinical criteria, the private physician began treatment with 5-iodothiouracil, which the patient took irregularly. Six months later, still exhibiting symptoms and signs of thyrotoxicosis, she was referred for I^{131} therapy. At this time the erythrocyte I^{131}-T_3 uptake was 24.0 per cent. The 24-hour thyroidal uptake of I^{131} was 67 per cent and the 48-hour serum PBI-I^{131} level was 0.43 per cent of the dose per liter (normal, less than 0.4 per cent). The symptomatic response to treatment with I^{131} was excellent, and the patient was judged to be euthyroid three months after therapy.

RESULTS

The results of the studies on Patient A.H. are summarized in Table 1. Except for the serum PBI level, all of the pre-treatment findings were consistent with the diagnosis of hyperthyroidism. The serum PBI level ranged from 5.1 to 5.8 μg. per 100 ml. on three separate occasions over a three-week period prior to therapy. Following radioiodine treatment, which was given on May 14, the PBI values showed a general downward trend, eventually reaching a constant level of approximately 4.0 μg. per 100 ml. seven months later when the patient was considered to be euthyroid. In September 1960, the concentrations of both butanol-extractable iodine (BEI) and PBI were determined in the same sample of serum. Both values were low and the difference between them was larger than the usual difference of 0.5 μg. per 100 ml. found in normal subjects.

The BMR and the serum cholesterol concentration returned to more normal levels after treatment in this patient, consistent with the clinical improvement.

The thyroidal I^{131} uptake also fell to normal following treatment, but the 48-hour serum PBI-I^{131} value remained elevated even after the patient had become eumetabolic, a finding noted by others following treatment of thyrotoxicosis (20). The erythrocyte uptake of I^{131}-T_3 decreased abruptly from 27 per cent before treatment to a normal level of 15 per cent two months following treatment, before the patient became eumetabolic.

Except for a transient unexplained rise in December 1960, the erythrocyte I^{131}-T_3 uptake remained normal thereafter.

A thyroxine degradation study was performed in December 1960. The rate of disappearance of I^{131}-thyroxine from the plasma increased slightly (half-time = 5.5 days; k = 12.6 per cent per day). However, because of an abnormally small extrathyroidal organic iodine pool (376 μg.), the calculated rate of thyroxine degradation was normal (47 μg. of iodine per day). This finding is consistent with the patient's eumetabolic state at that time.

Serum thyroxine-binding capacity was studied in Patient A.H. at

TABLE 2. SERUM PBI LEVEL AND THYROXINE-BINDING CAPACITY IN PATIENTS A.H. AND W.R., THEIR RELATIVES, AND CONTROL GROUPS

Subject	Relationship	Clinical status	Serum PBI (μg./100 ml.)	Thyroxine-binding capacity (μg./ml.)	
				TBG	TBPA
A.H.	(Propositus)	Hyperthyroid	5.1	0.08	1.22
W.R.	Sister	Hyperthyroid	6.6	0.11	1.35
		Partly treated	—	0.13	1.38
R.R.	Brother	Euthyroid	4.9	0.17	2.26
M.R.	Mother	Euthyroid	4.6	0.25	1.56
23 Normal subjects		Range	3.9– 7.6	0.13 – 0.25	1.30 – 2.70
		Mean ± S.D.	—	0.16 ± 0.02	2.04 ± 0.30
11 Hyperthyroid patients		Range	7.8–13.0	0.11 – 0.21	1.10 – 2.30
		Mean ± S.D.	—	0.15 ± 0.03	1.84 ± 0.39
Difference between means (norm *vs.* hyperthyroid)				0.01	0.20
S.E. of difference				0.01	0.13

approximately monthly intervals (Table 1). The initial values for TBG and TBPA were abnormally low. Following treatment with I^{131}, however, the concentrations of both these thyroxine-binding proteins approached the normal range, finally becoming stable at low-normal levels. There appeared to be an inverse relationship between the erythrocyte I^{131}-T_3 uptake and the level of thyroxine-binding proteins in this patient.

Table 2 shows the serum PBI and thyroxine-binding protein levels in Patient A.H., in her sister W.R., and in 2 other members of the family. The data pertaining to Patient W.R. were obtained on two occasions six months apart: 1) before any treatment, and 2) after an unsuccessful course of therapy with 5-iodothiouracil. The serum TBG capacity in this patient was in the low-normal range on both occasions, the difference between the two values being of doubtful significance. The pre-albumin value remained at a low-normal level.

A search was made for thyroxine-binding abnormalities among other members of this family. The findings are included in Table 2. The only other living sibling, a 35-year-old brother, was said to be in good health and had no history of thyroid disease according to his private physician. Serum was obtained and both the PBI level and thyroxine-binding pattern were found to be normal. The father of the patients was dead. The mother, who was available for examination, was 74 years old and manifested no evidence of thyroid disease. Her serum PBI and thyroxine-binding protein levels were normal.

Included in Table 2 are the control results obtained in 23 normal subjects and in 11 untreated hyperthyroid patients exclusive of the cases presented. These values for TBG capacity, both in the normal subjects and in the hyperthyroid patients, were somewhat lower than those obtained by other investigators, who found a mean TBG capacity among normal subjects of approximately 0.20 μg. per ml. (3, 11, 12). We have no explanation for this discrepancy. Nevertheless, in agreement with other published studies, the TBG capacity in our control hyperthyroid group was not significantly different from that in our normal group ($P > 0.3$).

The values obtained for the amount of thyroxine bound to pre-albumin may not represent the actual binding capacity, since we have found that at the thyroxine concentration used routinely in this study (4.50 μg. per ml.) the plateau of TBPA binding is approached but not yet reached. However, because all of the TBPA determinations were made at the same thyroxine concentration, a comparison between the patients in this study and normal subjects is probably valid. The TBPA levels so obtained were higher than the normal mean value of 1.10 μg. per ml. found by Ingbar and Freinkel using tris-maleate buffer (11). Furthermore, in contrast to their observation, we found only a small, questionably significant difference in TBPA values between the normal and the hyperthyroid patients ($P > 0.1$).

Figure 1 illustrates the quantitative abnormalities already noted in I^{131}-thyroxine binding by the serum proteins in Patient A.H. before and after treatment, and in her sister W.R. The serum thyroxine-binding pattern in a normal subject is included for comparison. The concentration of thyroxine in each of the 4 sera was approximately the same.

Table 3 shows the I^{131} compounds in the serum of each patient. In Patient A.H., a large tracer dose of I^{131} (500 μc.) had been administered at a time when she was considered to be euthyroid (January 1961). In Patient W. R. the analysis was made on serum collected two days after a therapeutic dose of I^{131}, given when she was still thyrotoxic. I^{131}-iodoprotein was present in both sera, comprising 11 and 17 per cent respectively of the total I^{131} (13 and 21 per cent of the organic I^{131}). The relative

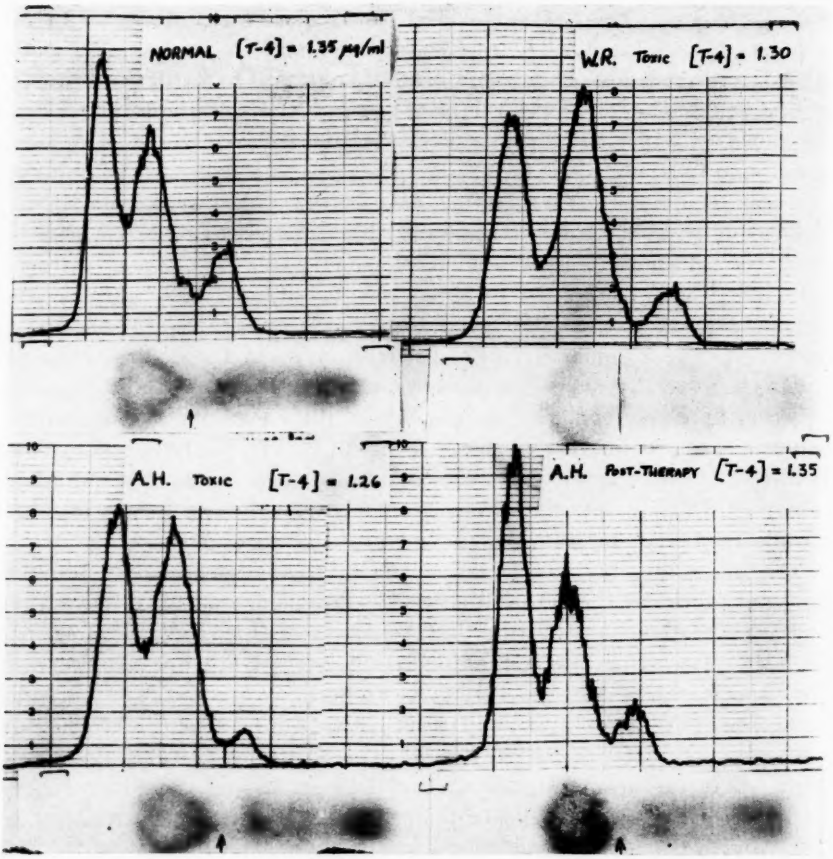

FIG. 1. Electrophoresis of I^{131} thyroxine-serum mixtures in a normal subject, in Patient W.R. (before I^{131} treatment) and in Patient A.H. (before, and six months after I^{131} treatment).

The total thyroxine concentration in each mixture was approximately the same and is noted in the figure. Paper strips stained for protein with bromphenol blue are shown beneath the radioactivity record. The origin is indicated by an arrow. The anode is on the left. Buffer:0.1 *M* ammonium carbonate, pH 8.4. Direction of buffer flow is to the right (reverse-flow method).

amounts of labeled iodotyrosine and iodothyronine were not significantly altered. The ratio of I^{131}-thyroxine to I^{131}-triiodothyronine was the same in both sera.

Further studies were carried out on the serum of Patient W.R., by means of starch block electrophoresis in barbital buffer (Fig. 2); this separated the radioactivity into two components corresponding to the

albumin and the alpha-globulin zones. Twenty-four per cent of the radioactivity eluted from the starch was butanol-insoluble and most of this material migrated with albumin. The proportion of butanol-insoluble radioactivity found by this method was in good agreement with the results obtained by column chromatography indicating that 21 per cent of the organic I^{131} was iodoprotein.

Figure 3 shows the results of ultracentrifugal analysis of a sample of serum (Patient W. R.) which was rendered iodide-free by preliminary resin treatment. The radioactivity sedimented at the same rate as the average serum protein. According to the results obtained by Robbins *et al.* (21), thyroglobulin would, under the conditions of this experiment, sediment faster than the average serum protein.

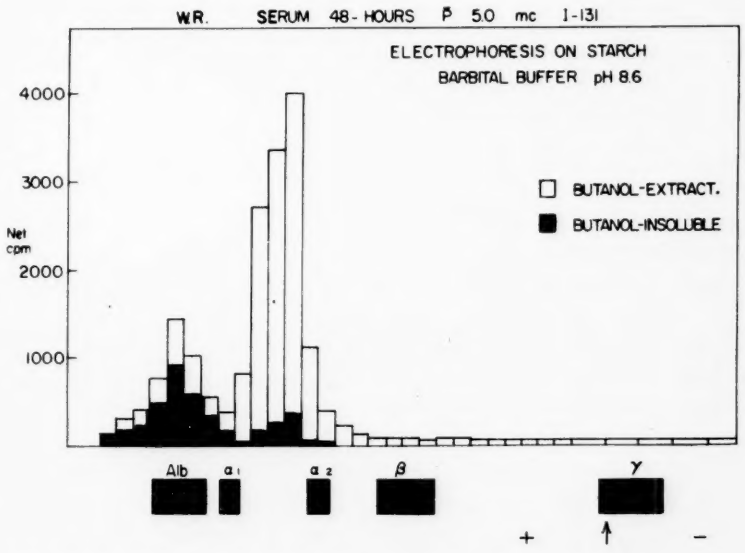

FIG. 2. Electrophoresis of serum from Patient W.R., forty-eight hours after ingestion of 5.0 mc. of I^{131}.

Supporting medium: unhydrolyzed potato starch (30×30 cm. block). Buffer: sodium barbital, pH 8.6, ionic strength 0.05. The 2 ml. of serum applied at the origin is indicated by the arrow. A potential of 6 volts per cm. was applied for eighteen hours at 4° C. At the end of the run the position of the proteins was determined by blotting the surface of the starch with a filter paper strip, which was then stained with bromphenol blue. The starch was cut into 0.5 -cm. sections, each of which was eluted with saline. The radioactivity eluted from each section is shown on the ordinate of the graph as the total height of the column. Each eluate was acidified to pH 3.0 with HCl and subjected to extraction three times with equal volumes of n-butanol saturated with 0.1 *N* HCl. The unextracted radioactivity is indicated in the graph by dark columns.

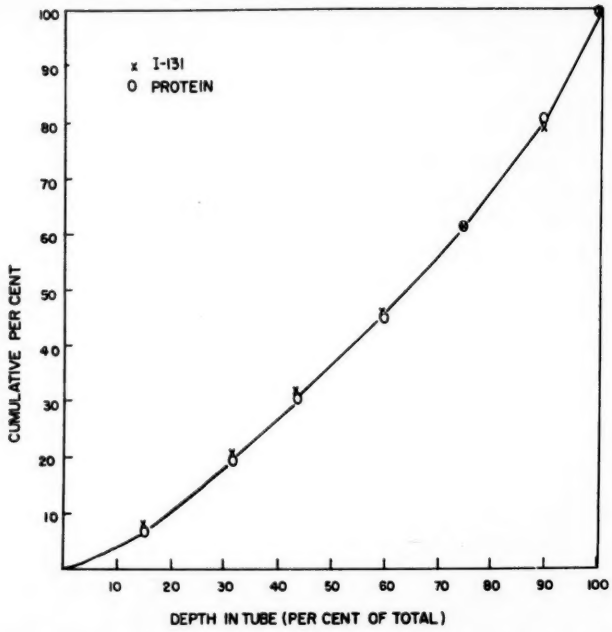

FIG. 3. Sedimentation in the preparative ultracentrifuge (Spinco Model L, No. 40 Rotor) of I^{131}-labeled compounds in the serum from Patient W.R. forty-eight hours following a 5.0-mc. dose of I^{131}.

As a preliminary treatment, an 8-ml. aliquot of serum was applied to a column of anion-exchange resin (Dowex 1×2, 100–200 mesh) equilibrated with 0.2 *M* ammonium acetate buffer, pH 5.6. The resin adsorbed all iodide and some of the iodothyronines. The I^{131} in the unabsorbed fraction was shown by paper chromatography (BDA) to consist of equal parts of iodothyronines and of origin material. This fraction (12 ml.) was sedimented in the ultracentrifuge for three hours at 39,000 rpm. Successive 1-ml. samples were removed, starting at the top of the tube. Radioactivity in each sample was measured in a well-type scintillation counter. Total protein was determined by ultraviolet absorption at 280 mμ.

DISCUSSION

The association of a normal serum PBI level and clinical thyrotoxicosis in these 2 patients is best explained on the basis of the observed changes in thyroxine-binding by serum proteins. In the presence of a normal level of protein-bound iodine, a reduction in the number of protein sites available for binding of thyroxine would be expected to result in an abnormally high concentration of free, metabolically active thyroxine.

This hypothesis assumes that the nature of the circulating hormone is not altered, and that it is predominantly thyroxine. Thyrotoxicosis due to an increased blood concentration of triiodothyronine relative to that of

thyroxine would be associated with a normal PBI level. An example of this situation has recently been described (23). In both of the present cases, however, the relative amounts of triiodothyronine and thyroxine were not very different from those in other cases of thyrotoxicosis (24). Although in Patient A. H. the analysis of the serum was carried out several months after radioiodine therapy, in her sister the analysis was made during a period of thyrotoxicosis.

The observed alteration in thyroxine-binding proteins of the serum would appear, therefore, to be significant in the cases under discussion. Since TBPA levels may be altered in many acute diseases, and were not significantly different in the present cases from the TBPA levels found in

TABLE 3. THE I^{131} COMPOUNDS IN THE SERUM OF PATIENTS A.H. AND W.R.

Patient	Dose of I^{131} (mc.)	Time of serum collection	Per cent of total I^{131}*				Ratio** $T_4:T_3$
			Iodoprotein	MIT, DIT	T_4 and T_3	Iodide	
A.H.	0.5	72 hrs.	11	1	70	18	11
W.R.	5.0	48 hrs.	17†	3	60	20	11

* Determined by column chromatography on Dowex 1×2. The method of Galton and Pitt-Rivers (22) was used with the following modifications: Resin was adjusted to pH 11 with NH_4OH before addition of serum (4 ml.) which was brought to pH 10 with NH_4OH. At this pH, all I^{131}-labeled compounds, except iodoprotein, are adsorbed to the resin. Iodotyrosines were then eluted with 2.7 *M* acetic acid. Iodothyronines were eluted with 10 *M* acetic acid. Iodide was determined by the difference between the radioactivity applied to the column and that recovered in the eluates.

** Determined by paper chromatography of the lyophilized column fraction. Solvent: n-butanol-1,4-dioxane (80:20) equilibrated with 2*N* NH_4OH (BDA). Radioactivity was measured by scanning and autoradiography.

† The radioactivity in this fraction was butanol-insoluble and remained at the origin on paper chromatography in BDA.

other patients with hyperthyroidism, it appears that the important feature of the cases being discussed is the reduction in serum TBG levels.

If we assume that a change in TBG capacity is brought about by a change in the concentration of the binding protein, we may speculate on possible mechanisms for such alterations. An increased rate of degradation of the binding protein secondary to hypermetabolism could account for a reduction in the serum TBG level. One would expect to find similar abnormalities, however, in other thyrotoxic patients or in any hypermetabolic condition, but TBG capacity has been found to be normal in such cases.

Urinary loss of the binding protein, such as may occur in certain cases of nephrosis, is not likely in the present cases, since proteinuria was not observed.

A decreased rate of synthesis of TBG in the face of a normal or increased

rate of degradation is another possibility. There is no evidence for or against this point. Measurement of the turnover rates of the thyroxine-binding proteins would bear directly on this question. Such studies must await the purification of these substances.

The observed increase in TBG capacity following treatment in Patient A.H. is difficult to evaluate, since there are few data on the effect of treatment on the level of TBG in other cases of hyperthyroidism. Incidentally, the observed decrease in the erythrocyte I^{131}-T_3 uptake appeared to be correlated with the rise in TBG capacity (Table 1)—a finding consistent with the view that the erythrocyte uptake is an indirect measure of the unoccupied sites on the binding protein.

Inhibition of thyroxine binding by a variety of substances has recently been investigated. Dinitrophenol, salicylates and diphenylhydantoin displace thyroxine from binding sites and lower the serum PBI level (25). Neither of the patients in this study was exposed to any substance known to inhibit thyroxine binding, however.

The presence of iodoprotein in the serum represents another interesting feature of these patients. In Patient W. R. it seems certain that this material was not thyroglobulin. In its electrophoretic mobility and sedimentation characteristics, it resembled the iodinated compound found in the blood of patients with functional thyroid carcinoma (26) and in certain cases of goitrous cretinism (27). Unfortunately this substance was not further characterized in the present instance, so we cannot define its role in the disordered physiology of our patients. Iodoproteins, electrophoretically similar to albumin, have been found in the serum of other patients with thyrotoxicosis following radioiodine therapy (28–30).

The sisters described in this report present, therefore, an unusual combination of abnormalities—hyperthyroidism, diminished binding of thyroxine by serum alpha-globulin, and an abnormal iodoprotein in the blood. It is tempting to ascribe all 3 of these abnormalities to an inherited defect. By what mechanism such a genetic factor could produce these manifestations is an intriguing but unanswered question.

Acknowledgments

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THE EFFECT OF PURIFIED BOVINE THYROID-STIMULATING HORMONE IN MAN. I. DOSE-RESPONSE CHARACTERISTICS STUDIED WITH I^{132}*

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ABSTRACT

The effect of a highly purified bovine thyrotropin (TSH) was studied in 12 human volunteers. The response was measured by the rise in the two-hour thyroidal uptake of I^{132}. Dose-response curves for purified TSH, and for a commercial preparation (Thytropar) given in equivalent units, did not differ significantly. After TSH stimulation, the thyroidal I^{132} uptake following intravenous administration of I^{132} showed an increase of borderline significance compared with the uptake following oral administration, whereas control uptakes did not differ significantly for the two routes. The refrigerated purified preparation of TSH maintained its potency for as long as a month after reconstitution.

BOVINE thyroid-stimulating hormone (TSH) purified by the method of Pierce *et al.* (1, 2) has recently become available in limited quantity for experimental purposes. Prior to extensive use, it was felt advisable to learn more about its behavior when given to human subjects. This paper reports a dose-response study in humans comparing this substance (hereafter referred to as "purified TSH") with a commercially available relatively crude bovine TSH. It includes data on the lasting qualities of purified TSH when reconstituted, and a comparison of the responses to TSH in terms of the thyroidal uptake of I^{132} after oral and intravenous doses of the isotope.

SUBJECTS AND METHODS

All 12 subjects were healthy young college students—paid volunteers. None had evidence of thyroid disease or of excess iodine ingestion. All had normal levels of serum protein-bound iodine (PBI). The response to a dose of TSH was measured by its effect on the two-hour thyroidal uptake of a dose of I^{132} given twenty-one to twenty-two hours after the TSH. At least one determination of the two-hour thyroidal I^{132} uptake was made prior to administration of TSH. In more prolonged studies, these control determinations were repeated at intervals. Generally, three doses of TSH were given weekly—on Sunday,

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Tuesday and Thursday—followed by uptake measurements on Monday, Wednesday and Friday.

For the dose-response study, each subject received intramuscularly 0.003, 0.009 and 0.027 unit per Kg. of body weight of purified TSH and of commercial TSH (Armour's Thytropar). These 6 doses (at three levels for each of the two drugs) were given in a randomly assigned, counterbalanced order, arranged so as to eliminate the systematic influence of the order of administration.

Twelve subjects (11 male and 1 female) participated in the dose-response study. Six were given the I^{132} intravenously, and 6 were given it orally. The two groups of 6 subjects each, were handled separately and were scheduled according to separate counterbalancing schemes. After completion of the 6 doses of TSH, 7 subjects (3 who had originally received the I^{132} intravenously, and 4 who had received it orally) were further studied after administration of I^{132} by the alternate route. Control thyroidal uptakes were again measured, giving I^{132} by the new route. Then each subject received 2 doses of TSH (one purified, one commercial), each followed by an I^{132} uptake measurement; a dose of 0.027 unit per Kg. of body weight was used for both the purified TSH and the commercial product. The order of administration was rotated.

One male subject receiving I^{132} orally and one female subject receiving it intravenously were given a constant dose of purified TSH at varying times after reconstitution with physiologic saline.

The I^{132} was extracted just prior to use by elution (with 0.01 *M* NH_4OH) of a column of tellurium[132] adsorbed onto alumina (3). The columns were provided biweekly by Brookhaven National Laboratories. The activity of the I^{132} solution thus obtained was estimated in a source calibrator. The approximate dose was 7 μc. The exact dose was determined by use of a standard consisting of 1 ml. of the solution pipetted into a beaker and counted in a position mimicking that of the thyroid gland, using the same scintillation-detection equipment employed for the uptake measurements.

As uptakes were being determined repeatedly, and contamination of the I^{132} solution with I^{131} could not be avoided completely, background counts were made over the neck and thigh before each dose of I^{132} was given. In no case did the thigh count rise significantly above the background count, nor did residual radioactivity over the neck exceed 10 per cent of the room background. Uptakes were calculated as the difference between the two-hour counts over the neck and the thigh (minus their respective pre-I^{132} residual counts) taken as a percentage of the amount administered. The neck-thigh ratio (4) was also calculated, but not used in the final analyses, as its coefficient of variation was greater than that of the percentage uptake. All counts were corrected for radioactive decay.

TSH was prepared from frozen bovine anterior pituitaries by methods employing chromatography on DEAE cellulose and then on carboxymethyl cellulose (1, 2). When subjected to starch-gel electrophoresis, the preparation exhibited only the bands identified as b and c. Activity assayed in chicks by the P^{32} method of Greenspan *et al.* (5) was approximately 15 USP units per mg. Human serum albumin (15 mg. per mg. of TSH) was added to prevent loss of active protein on glassware. The preparation was dissolved in water, sterilized by filtration, and lyophilized. Prior to use it was redissolved in physiologic saline. Administration was by the intramuscular route. TSH which had been maintained in solution under refrigeration as long as one month was tested in this manner.

RESULTS AND COMMENTS

The dose-response curves calculated for the two forms of TSH are shown

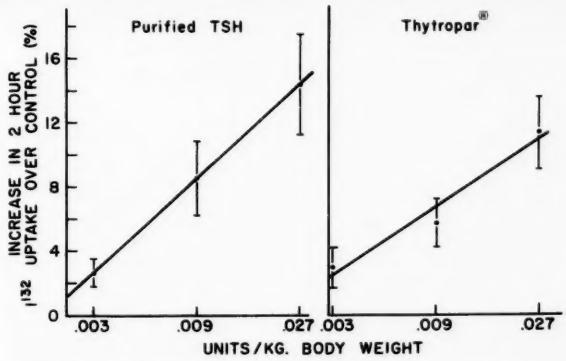

FIG. 1. Log dose-response curves for purified bovine TSH (left) and for Thytropar (right).

in Figure 1. These represent pooled data from all 12 subjects. The statistics on which these curves are based are presented in Table 1. Although the slope for Thytropar was somewhat flatter than that for the purified preparation, the difference was not statistically significant. This suggests that no major inhibitor is present in the less pure preparation. Furthermore, there was no significant difference between the levels of response to similar doses (in units) of the two preparations. As these unitages were initially selected on the basis of animal assays (chick P^{32} uptake for the purified preparation, and chick I^{127} release for Thytropar), this finding indirectly confirms the applicability of these assay results to human subjects.

The data on I^{132} intravenously were treated separately from the data on I^{132} orally, in three-way analyses of variance, testing for significant differences between subjects, between sessions, and between doses of TSH. The results are shown in Table 2. The TSH-dose differences were highly significant. In addition, there was a significant difference between the

TABLE 1. STATISTICS OF DOSE-RESPONSE REGRESSION LINE DERIVED FROM POOLED DATA ON 12 SUBJECTS*

	Purified TSH	Thytropar
Equation of line	$Y = -3.208 + 12.208X$	$Y = -1.758 + 8.794X$
Standard deviation of Y	3.288	2.567
Lambda	0.269	0.292
Mean and S.E. of increase in uptake when dose is:		
.003 unit/Kg.	2.642 ± 0.860	2.917 ± 1.274
.009 unit/Kg.	8.508 ± 2.330	5.675 ± 1.543
.027 unit/Kg.	14.233 ± 3.079	11.308 ± 2.264

* Y = increase in % I^{132} uptake; X = log dose (unit/Kg. $\times 10^3$).

TABLE 2. ANALYSIS OF VARIANCE FOR EFFECTS OF TSH ON THYROIDAL I^{132} UPTAKE, TREATING SEPARATELY THE SUBJECTS TESTED WITH I^{132} ORALLY AND INTRAVENOUSLY*

	Sum of squares	df	Mean square	F	P
			I^{132} Intravenously		
Between subjects	461.83	5	92.366	3.529	.025
TSH-dose	1454.21	5	290.842	11.112	.001
Between sessions	260.68	5	52.136	1.992	>.10
Residual	523.46	20	26.173		
			I^{132} Orally		
Between subjects	478.683	5	95.737	20.262	.001
TSH-dose	181.746	5	36.349	7.693	.001
Between sessions	18.913	5	3.783	0.801	>.10
Residual	94.501	20	4.725		

* The data are expressed as % response uptake minus % control uptake.

responses of individual subjects. However, the counterbalanced dosage schedule was designed to eliminate any systematic effect of individual differences on the dose-response curve. No significant intersession differences were noted.

The wide variation in individual responsiveness, and the fact that those subjects who were tested intravenously tended to be more responsive than those tested orally, led to the speculation that the TSH-stimulated gland might be especially avid for iodide at the beginning of the uptake curve. Thus the initial high blood level of radioiodine immediately after intravenous injection of I^{132} would lead to a disproportionately high two-hour thyroidal uptake after TSH stimulation. This hypothesis was examined by repeating a portion of the study in the same subjects, giving I^{132} by the alternate route. The results are presented in Table 3. Control thyroidal

TABLE 3. COMPARISON OF THYROIDAL I^{132} UPTAKES (% DOSE) AFTER ISOTOPE GIVEN BY ORAL OR INTRAVENOUS ROUTE

Subject	Original route of admin.	Control		Purified TSH (.027 unit/Kg.)		Thytropar (.027 unit/Kg.)	
		I.V.	Oral	I.V.	Oral	I.V.	Oral
A	Oral	9.9	8.1	15.1	18.5	21.2	15.5
B	Oral	8.9	11.3	17.4	12.2	15.3	14.4
C	Oral	5.4	10.5	33.3	28.5	24.5	20.8
D	Oral	10.2	9.1	11.4	9.6	9.3	10.6
E	I.V.	16.0	9.2	55.6	24.6	41.9	29.4
F	I.V.	15.3	12.3	32.9	26.0	24.3	20.0
G	I.V.	16.2	10.4	38.6	34.9	35.7	24.6
Mean difference, I.V. *vs.* oral		+1.57		+7.57		+5.27	
t		0.979		1.889		2.242	
P		.10<P<.25		.05<P<.10		.025<P<.05	

uptakes did not differ significantly by the two techniques. This confirms the statement by Halnan and Pochin (6) that reliable I^{132} uptake results may be obtained in as short a period as two hours, using the oral route (in fasting subjects).

After TSH stimulation the thyroidal I^{132} uptake was usually greater following administration of I^{132} by the intravenous route than by the oral route. However, these differences were only of borderline significance, since that for purified TSH failed to meet the criteria for statistical significance and that for Thytropar was significant only at the 5 per cent

TABLE 4. EVALUATION OF LASTING QUALITIES OF THE PURIFIED TSH

Date	Age and dose of TSH preparation	Thyroidal I^{132} uptake (% dose)
Female Subject No. 1—I^{132} Intravenously		
March 2	Control	12.3
3– 4	Fresh TSH, .009 U/Kg.	16.3
6– 7	3-day-old TSH, .016 U/Kg.	22.2
8– 9	7-day-old TSH, .009 U/Kg.	14.75
10–11	9-day-old TSH, .009 U/Kg.	18.9
15–16	14-day-old TSH, .009 U/Kg.	16.1
18	Control	10.23
23	22-day-old TSH, .016 U/Kg.	20.0
March 25	1-month-old TSH, .016 U/Kg.	20.5
Male Subject No. 2—I^{132} Orally		
March 18	Control	8.91
21	Fresh TSH, .009 U/Kg.	16.1
23	Fresh TSH, .009 U/Kg.	13.7
25	2-week-old TSH, .009 U/Kg.	13.3
28	2-week-old TSH, .009 U/Kg.	14.9
30	1-month-old TSH, .009 U/Kg.	15.9
Apr. 1	1-month-old TSH, .009 U/Kg.	15.9

level. Thus, on the basis of these findings, we cannot conclude that there is increased avidity for iodide in the TSH-stimulated gland early in the I^{132} uptake curve. Further studies, with a larger sample, are necessary before this question can be settled. The wide differences noted in our original study between the responsiveness of the two groups is apparently largely due to individual variations within the groups rather than to the route of I^{132} administration.

The purified TSH which had been kept in physiologic saline solution under refrigeration for as long as one month showed no decrease in effectiveness in the 2 subjects tested (Table 4).

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THE EFFECT OF PURIFIED BOVINE THYROID-STIMULATING HORMONE IN MAN. II. LOSS OF EFFECTIVENESS WITH PRO-LONGED ADMINISTRATION*

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ABSTRACT

Four normal human subjects received repeated doses of a highly purified bovine thyrotropin (prepared by the method of Pierce *et al.*) and the response was monitored by the rise in the two-hour thyroidal uptake of I^{132}. All subjects initially responded to doses of TSH varying from 0.0032 to 0.050 unit per Kg. of body weight. All became refractory to the effects of the TSH after five to twenty-nine doses. Sera obtained after development of resistance contained hemagglutinating and neutralizing antibodies to thyrotropin.

WHEN preparations of thyrotropin (TSH) of relatively low purity have been administered for prolonged periods to animals or man, their effectiveness has regularly declined after a variable period of treatment (1–5). Now that a highly purified preparation of bovine TSH is available, it is possible to examine more closely the extent to which impurities cause the development of refractoriness. The studies reported here demonstrate that resistance to a highly-purified preparation does develop regularly in normal human subjects. The sera of these resistant subjects were then examined for the presence of circulating antibodies, and the specificity of these antibodies for TSH was evaluated.

SUBJECTS AND METHODS

Four healthy male college students, paid volunteers, were studied. None had objective evidence or a history of thyroid abnormality or of excessive ingestion of iodine. All had normal baseline levels of serum protein-bound iodine (PBI) and thyroidal I^{132} uptake.

Prior to the use of TSH, five two-hour I^{132} thyroidal uptake measurements were made daily as control observations. The subjects then generally received three intramuscular doses of TSH per week, each followed after twenty-one to twenty-two hours by a dose of I^{132} for measurement of the two-hour thyroidal uptake. When this schedule was un-

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avoidably interrupted, it was resumed as soon as possible after the point of interruption. Every two to three weeks during the study, and at the end, the preceding dose of TSH was omitted in order to recheck the control I^{132} uptake. Serum PBI concentration was determined by the method of Barker *et al.* (6), before TSH administration, toward the middle of the study, and after development of resistance.

The methods of preparation of the TSH and the I^{132} have been described in the foregoing paper (7). Prior to each dose of I^{132} for uptake measurement, background counts were made over the neck and thigh to detect residual radioactivity. In no case did the thigh count rise significantly above the background count, nor did that over the neck exceed the room background by more than 10 per cent. A dose of approximately 7 μc. of I^{132} was given orally for each uptake measurement. The difference between the net increase in neck and thigh counts at two hours, taken as a percentage of the radioactivity administered (corrected for radioactive decay) was calculated as the uptake value.

The maximal total dose of I^{132} administered to any subject was 266 μc. With the use of the I^{132}/I^{131} ratio reported by Halnan and Pochin (8), it can be estimated that the radiation to the thyroid from this dose corresponds to that from approximately 2.5 μc. of I^{131}.

The initial dose of TSH was .0063 unit per Kg. of body weight—a level shown by Einhorn (9) to cause a rise in the thyroidal I^{132} uptake, but no rise in the serum PBI level and no clinical symptoms. Two injections of this dose were given. The dose was then adjusted by doubling or halving until the minimal effective dose was determined. This was defined as that dose which on two successive occasions produced a thyroidal I^{132} uptake greater than two standard deviations above the mean of the pre-treatment uptakes. Once the dose which produced such an effect was reached, it was maintained until no longer effective. When a given dose had become ineffective, it was again doubled to see whether the effect would be renewed.

Sera obtained from all 4 subjects after development of resistance were examined by one of us (S.C.W.) for their ability to neutralize purified bovine and partially purified human TSH, and for the presence of hemagglutinating antibodies to a commercial bovine TSH preparation (Thytropar). The assays were conducted after repeated absorption of the subjects' sera with bovine serum and with luteinizing hormone. In the neutralization assay, the effectiveness of TSH was measured by its ability to release I^{131} from the thyroid-suppressed gland in the mouse (10). The presence of neutralizing material was evaluated by its ability to prevent this response to TSH. The procedural details have been published elsewhere (11). Hemagglutinating antibodies were assayed by a modification of the method of Boyden (12); details are to be published elsewhere.

RESULTS

The effect of TSH on thyroidal I^{132} uptake is summarized in Table 1. Figure 1 illustrates the plan of study, and the results for 1 subject. The minimal effective doses were .0031, .0063, .0250 and .0500 unit per Kg. of body weight in Subjects H, C, L and B, respectively. Three of the subjects (B, H and L) became refractory to this dose after five to thirteen doses over periods of ten to thirty-one days. When the dose of TSH was then doubled, no further rise occurred in Subjects B and L. Subject H showed a significant rise in uptake when the dose was first doubled, but then became refractory to this dose and to subsequent higher ones. Subjects B

TABLE 1. TWO-HOUR I^{132} THYROIDAL UPTAKE (% DOSE) IN RESPONSE TO A DOSE OF TSH 21–22 HOURS PREVIOUSLY

Date	Subject C		Subject H		Subject L		Subject B	
	mean, 5 controls 10.24 mean of controls .628 mean +2 11.50		8.57 .792 10.15		11.63 1.815 15.26		7.30 2.152 11.60	
	TSH dose	I^{132} uptake	TSH dose	I^{132} uptake	TSH dose	I^{132} uptake	TSH dose	I^{132} uptake
Apr. 11 or 12	.0063	14.48	.0063	14.51	.0063	13.46	.0063	10.98
13	.0063	13.42	.0063	12.77	.0063	11.64	.0063	8.37
15	.0031	7.72	.0031	10.44	.0125	15.69	.0125	10.58
18	.0031	11.58	—	—	.0125	11.86	.0125	9.18
20	.0031	12.40	0031	11.45	.0125	13.07	.0125	9.56
22	.0063	9.65	.0031	10.38	.0250	19.39	.0250	9.98
25	.0063	10.15	—	—	.0250	14.70	.0250	9.99
27	.0125	—	.0031	9.36	.0250	18.05	.0500	15.02
29	.0125	12.38	.0031	8.05	.0250	18.82	.0500	12.69
May 2	.0125	13.97	.0063	14.66	.0250	15.91	.0500	9.87
4	.0125	18.15	.0063	15.28	.0250	18.99	.0500	9.40
6	control	9.42	control	7.95	control	9.75	control	6.30
9	.0063	12.04	—	—	.0250	14.27	.1000	6.98
11	.0063	12.77	.0063	8.50	.0250	15.66	.1000	7.73
13	.0063	12.42	.0063	7.89	.0250	12.22	.1000	6.50
16	.0063	12.95	.0063	8.33	.0250	11.96	.1000	5.91
18	.0063	13.39	.0125	7.14	.0250	10.65	.1000	6.79
20	.0063	11.57	.0125	7.37	.0250	8.45	control	5.81
23	.0063	10.63	—	—	.0500	8.24	control	8.33
25	.0063	12.88	.0250	6.46	.0500	10.51	.1 A*	9.14
27	.0063	12.87	.0250	6.91	.0500	6.61	.1 A	8.04
31	—	—	—	—	control	13.71†	—	—
June 1	.0063	12.67	—	—	.05 A	10.94	.1 A	10.31†
3	.0063	12.73	—	—	.05 A	18.23	control	8.59
6	.0063	—	—	—	.05 A	9.50‡	control	4.44
8	.0063	12.29	—	—	—	—	—	—
10	.0063	12.08	—	—	control	11.23	—	—
20	control	10.96			control	9.86		
22	.0063	14.11			.05 A	12.47		
27	.0063	10.05§						
29	.0063	—	†					
July 6	.0063	12.09						
11	.0063	9.75						
13	.0063	10.89						
18	.0063	8.88						
20	.0063	11.95						
27	control	10.53†						
July 28	control	12.30						

* A = Armour's Thytropar.
† Date of blood for antibody determination.
‡ Three-hour uptake.
§ Ate a candy bar.

and L were given Thytropar[1] in comparable doses after resistance to the purified TSH was established. Both subjects showed some rise in thyroidal I^{132} uptake, though not to the levels originally reached with smaller doses of purified TSH. In one, the rise after administration of Thy-

[1] Thytropar (Armour) is a relatively crude bovine TSH preparation, containing approximately 1 USP unit per mg.

tropar was significant by the *t* test at the 2 per cent level; in the other the rise was not statistically significant.

Subject C continued to show a response to a dose of .0063 unit per Kg. for twenty-nine doses given over ninety-one days. He then failed to respond to three of four subsequent doses and presumably had become resistant. Time did not permit trial of increased dosage in this subject.

Subject H, who was originally the most sensitive to TSH stimulation, was the first to exhibit resistance, after a total dosage of only .0219 unit

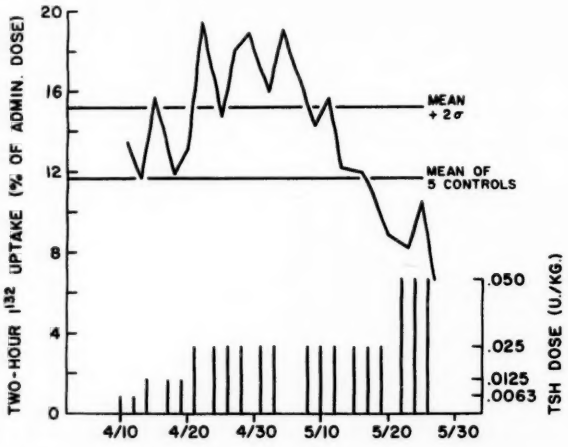

FIG. 1. Plan of study, and results for Subject L.

per Kg. Among the other 3 subjects, resistance developed after a latent period which was inversely related to the size of the dose originally required for response, so that the total dosage prior to resistance was comparable: that is, .1979 unit per Kg. for twenty-nine doses in Subject C; .2501 unit per Kg. for thirteen doses in Subject L; and .2001 unit per Kg. for nine doses in Subject B.

Serum PBI concentration did not change during the course of the study, except in Subject L in whom it fell to 3.1 μg. per 100 ml. shortly after development of resistance; four months later it was 7.0 μg. per 100 ml. In the other 3 subjects there was no significant change in PBI after development of resistance.

Sera from all subjects, obtained when clinical resistance to purified TSH had been established, showed hemagglutinating antibodies to Thytropar (Table 2). All sera likewise neutralized the I^{131}-releasing activity of highly purified bovine TSH and of human TSH (Table 3). Serum neutralization of human TSH was apparently less complete than that of bovine

TABLE 2. END-POINT OF TITER FOR HEMAGGLUTINATING ANTIBODIES TO THYTROPAR

Date (1960)	Control	Subject				Rabbit
		B	L	C	H	
Aug. 25	—	1:500	1:500	0	0	1:32,000
Aug. 30	—	1:1000	1:2000	1:1000	1:500	—
Sept. 16	0	—	1:1000	1:500	1:250	1:16,000
Oct. 10	—	—	—	0	1:500	1:8000

TSH; however, larger amounts of human TSH were used in the assay. By both techniques, higher antibody levels were detected in the sera of Subjects B and L than of Subjects C and H. It is of interest that Subjects B and L originally required relatively large doses of TSH in order to obtain a significant rise in thyroidal I^{132} uptake.

DISCUSSION

Decreased effectiveness of crude TSH after repeated administration to experimental animals was reported in 1934 by Hertz and Kranes (1) and by Friedgood (2), who also noted a temporary return of effectiveness when a newly prepared batch of TSH was introduced. Thompson *et al.* (3) found that 23 of 29 human subjects who were initially responsive became refractory to TSH after nine to forty-three days. In the remaining 6 pa-

TABLE 3. TSH-ANTI-TSH RESULTS WITH SERA OBTAINED AFTER DEVELOPMENT OF CLINICAL RESISTANCE TO TSH (MCKENZIE ASSAY)

Subject	Date (1960)	No. of mice	Dose of serum (ml.)	TSH	Control	Blood radioactivity 2 hrs. after injection (% of preinjection value)	S.D.	P
				Type & dose				
B	Aug. 30	4	0.5	PTSH*, 0.1 mu		83	± 6	.001
		4		PTSH, 0.1 mu		227	±85	
		4			Saline, 0.2 ml.	120	±11	
	Sept. 2	4	0.4	HTSH†, 0.25 mu		342	±56	.1
		4		HTSH, 0.25 mu		397	±83	
		4			Saline, 0.2 ml.	101	±17	
L	Aug. 30	4	0.5	PTSH, 0.1 mu		98	± 6	.001
		4		PTSH, 0.1 mu		227	±85	
		4			Saline, 0.2 ml.	120	±11	
	Sept. 2	4	0.5	HTSH, 0.25 mu		196	±23	.001
		4		HTSH, 0.25 mu		397	±83	
		4			Saline, 0.2 ml.	101	±17	
C	Aug. 30	4	0.5	PTSH, 0.1 mu		105	±15	.001
		4		PTSH, 0.1 mu		227	±85	
		4			Saline, 0.2 ml.	120	±11	
	Sept. 2	4	0.5	HTSH, 0.25 mu		270	±36	.001
		4		HTSH, 0.25 mu		397	±83	
		4			Saline, 0.2 ml.	101	±17	
H	Aug. 31	4	0.5	PTSH, 0.1 mu		99	± 3	.001
		4		PTSH, 0.1 mu		246	±51	
		4			Saline, 0.2 ml.	95	±22	
	Sept. 2	4	0.5	HTSH, 0.25 mu		193	± 2	.001
		4		HTSH, 0.25 mu		397	±83	
		4			Saline, 0.2 ml.	101	±17	

* PTSH = Purified TSH.
† HTSH = Human TSH.

tients, treatment, though still effective, was discontinued after ten to forty days. The development of resistance to bovine TSH in patients with pituitary insufficiency has been confirmed (4, 5).

Andersen and Collip (13) and Collip and Andersen (14) demonstrated that the serum of animals immunized to TSH transmits its inhibitory effect to other members of the same or other species. They also produced evidence (13, 15) that such serum, when administered for prolonged periods, caused a drop in the basal metabolic rate in normal animals. Scowen and Spence (16) showed that an inhibiting effect was transmitted by normal rabbit or human serum, but that serum from an immunized rabbit inhibited the TSH effect much more consistently. Thompson (17), using serum from a collie which had become highly resistant to sheep pituitary extract, demonstrated inactivation in guinea pigs of the effects of a variety of TSH preparations. On the other hand, Hohlweg and Dörner (18) recently demonstrated species specificity in the resistance of rats to hog, cattle and guinea-pig TSH.

Werner (19, 20) found that, in guinea pigs, refractoriness developed infrequently after treatment with a preparation of TSH presumed to be partially purified. He concluded that the phenomenon of refractoriness was an immunologic one and suggested that antibody formation might be in antagonism to associated proteins rather than to TSH itself. Recently, however, Werner and his collaborators (11) produced in rabbits precipitating and neutralizing antibodies to highly purified bovine TSH preparations, with evidence that antibody formation was probably specific for TSH. Cline *et al.* (21), using immunologic techniques as well as measurement of the thyroidal I^{131} uptake in mice, demonstrated that serum from rabbits immunized against bovine TSH specifically inhibited TSH activity and did not cross-react with other pituitary hormones.

The work reported here indicates that, with use of a highly purified preparation of bovine TSH in repeated minimally effective doses, refractoriness to its action develops regularly in human subjects after a time interval comparable to that found for less pure preparations. When this refractoriness develops, an increase of the TSH dose does not overcome it. After refractoriness developed, the sera from all subjects contained hemagglutinating antibodies to TSH. Since these were demonstrated after absorption of the sera with beef serum and with luteinizing hormone, it seems probable that the antibodies were specific for TSH.

Sera from the TSH-resistant subjects neutralized human TSH as well as purified bovine TSH. Although other interpretations are possible, this finding suggests that the neutralizing material reacted to TSH itself rather than to a foreign, species-specific protein. If this be true, refractori-

ness would also be expected to apply to endogenous TSH, with eventual development of myxedema during continued administration. Further evaluation of this point would be facilitated by the availability of highly purified human TSH for study in human subjects.

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PROGNOSTIC VALUE OF EARLY DETERMINATION OF THYROIDAL RADIOIODINE UPTAKE FOLLOWING I^{131} THERAPY

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ABSTRACT

With the aid of I^{132} (half-life, 2.3 hours) the thyroidal radioiodine uptake was measured in 41 cases of hyperthyroidism soon after I^{131} therapy, without delivering large doses of radiation to the thyroid gland. The prognostic value of a thyroidal uptake test soon after I^{131} therapy is very limited. It is unlikely that the patient will be cured unless the uptake has decreased three weeks after the treatment, though depression of the uptake at that time was observed even in a large proportion of cases that later required more therapy. In patients requiring retreatment, the uptake, if depressed, showed a tendency to recover between three weeks and two months after therapy. In most cases of remission there was a tendency toward further reduction of the thyroidal radioiodine uptake between three weeks and two months after I^{131} treatment.

THE aim, in radioiodine treatment of thyrotoxicosis, is to effect a cure with a single therapeutic dose; if this fails, further treatment is usually given after two to four months. The long interval between doses is necessary because the clinical response may be delayed for a considerable length of time after the radiation dose has been delivered to the thyroid gland. Complete remission may, therefore, take more than one year, notably in patients who require repeated doses of I^{131}. Several authors have discussed the possibility of determining the early radiation effects on the thyroid and of thus shortening the interval between successive doses. Myant (1) investigated the "neck/thigh ratio" two hours after an I^{131} tracer dose, both prior to and three weeks after I^{131} therapy; in view of the high residual radioactivity in the body, the tracer doses contained up to 530 μc. Larsson (2) determined the radioactivity in the urine twenty-four hours after an I^{131} tracer dose administered two weeks, and twenty to forty days after I^{131} therapy. The behavior of the therapeutic dose itself may also afford information of prognostic value (3, 4).

A direct tracer measurement of the thyroidal uptake can be made soon

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after I^{131} treatment, provided a large enough tracer dose is administered. A tracer dose of 500 μc. of I^{131} is generally required to insure a statistically adequate number of counts in excess of the background count from the remains of a therapeutic dose given three weeks earlier (4). Doses of that order of magnitude may have some added therapeutic effect. Commensurate doses of iodine isotopes with shorter half-lives can be administered, however, without fear of an undesirable radiation effect on the thyroid gland. With the aid of I^{132} (half-life, 2.3 hours), we studied the thyroidal radioiodine uptake three weeks after I^{131} therapy.

SUBJECTS AND METHODS

The investigation was conducted in the period 1956 through 1959 in 41 patients with clinically unequivocal hyperthyroidism, 38 of whom had diffuse or nonpalpable goiter and 3, toxic nodular goiter. All of them were treated as outpatients.

The thyroidal uptake was measured with a scintillation counter, provided with a 12°-angle conical lead collimator. The crystal-to-skin distance was 30 cm. No pulse height analyzer was used, but the influence from secondary radiation was reduced by a 2-mm. lead filter.

The tracer dose of I^{132} was injected intravenously and the counting rates over the neck were continuously recorded by a counting rate meter combined with an automatic pen writer (5). The difference between the counting rates at one minute and eleven minutes after the injection was, after correction for physical decay of I^{132} (6), expressed as a percentage of the counting rates obtained from a standard solution of I^{132} with the same activity as that of the administered tracer dose. This value, termed *H* (the initial accumulation rate), is also influenced by changes in extrathyroidal radioiodine within the volume of tissue scanned. The test, however, makes possible satisfactory discrimination between hyperthyroid and euthyroid patients (5, 6). Under the measuring conditions used in this investigation, approximately 95 per cent of euthyroid patients showed an *H* value of less than 5 per cent, and approximately 95 per cent of hyperthyroid patients, an *H* value exceeding 5 per cent (6).

The *H* value was determined in all patients prior to, and also three weeks and two months after I^{131} therapy. Three weeks after the I^{131} therapy it was possible to determine *H* in most patients following administration of less than 400 μc. of I^{132}. A few patients received doses of 400–1000 μc.

The patients underwent repeated follow-up examination at intervals of two to four months after I^{131} therapy. The first follow-up examination included clinical evaluation, determination of the serum protein-bound iodine (PBI) and cholesterol levels, the 24-hour thyroidal I^{131} uptake, the 24-hour urinary I^{131} excretion, and usually the basal metabolic rate. A remission was assumed to have occurred if the patient was distinctly improved at the time of examination two or three months after treatment, and was euthyroid or hypothyroid at subsequent follow-up visits. Otherwise treatment was repeated two to six months (average, 3.8 months) after the last dose. At the time of writing, the total follow-up time varied between nine months and four years after the I^{131} dose being studied.

RESULTS

Of the 41 patients treated, 20 were cured of hyperthyroidism by the I^{131} dose concerned in this investigation; 21 patients required retreatment.

Three weeks after the I^{131} therapy, depression of the thyroidal radioiodine uptake was observed in 19 patients who were in a state of remission at the time of follow-up examination, and in 17 who required re-treatment. The reduction was, on the average, greater in those with remissions than in those requiring re-treatment (Fig. 1). Normal *H* values were observed as early as three weeks after the treatment in 14 of the 20 patients exhibiting

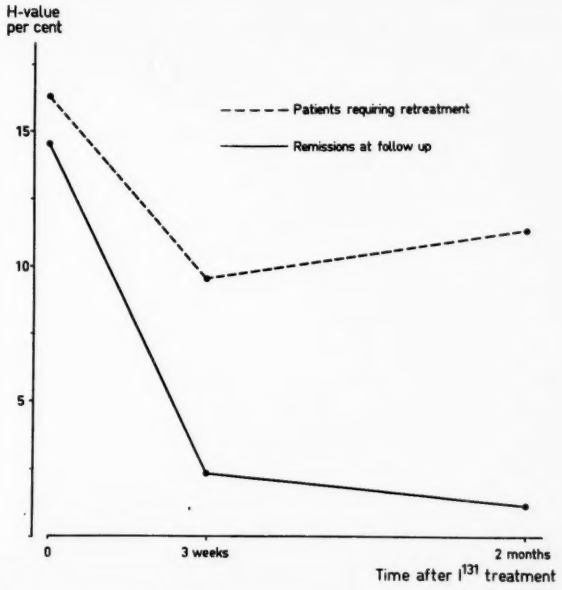

FIG. 1. Radioiodine uptake by the thyroid gland before and after I^{131} treatment. Each point on the solid line represents the mean for 20 patients with remissions; each point on the broken line represents the mean for 21 patients requiring re-treatment.

remission at the time of follow-up, but they were also found in 7 of the 21 who required re-treatment (Table 1).

In 21 patients requiring re-treatment, the *H* values, if depressed three weeks after therapy, showed a tendency towards restoration. From three weeks to two months after I^{131} therapy the initial accumulation rate (*H* values) rose by 2 per cent in 11 of these 21 patients. No rise of similar magnitude was observed in any of the patients with remissions at the time of follow-up. On the contrary, in most cases of remission there was a tendency toward a further decrease in the thyroidal radioiodine uptake from three weeks to two months after I^{131} therapy (Fig. 1). Hence the test results two months after treatment were far more consistent with the clinical findings than were the test results three weeks after treatment

(Table 1). However, three weeks after treatment, none of the patients found to be in a state of remission at the time of follow-up had *H* values exceeding 10 per cent, whereas half of the cases requiring re-treatment at that time had *H* values higher than 10 per cent (Table 1).

DISCUSSION

I^{132} with its short half-life enables the thyroidal radioiodine uptake to be studied soon after I^{131} therapy. Notwithstanding the higher radiation energies of I^{132}, its short half-life results in a substantially smaller total radiation dose. The radiation dose in the thyroid after administration

TABLE 1. RELATION OF *H* VALUES THREE WEEKS AND TWO MONTHS AFTER I^{131} TREATMENT TO THE CLINICAL RESULTS OBSERVED AT THE TIME OF FOLLOW-UP EXAMINATION

Time after I^{131} treatment	*H* value (%)	No. of remissions at time of follow-up exam.	No. of patients requiring re-treatment
3 wks.	>5 (elevated)	6	14
	<5	14	7
2 mos.	>5 (elevated)	2	17
	<5	18	4
3 wks.	>10	0	10
	<10	20	11

of I^{132} will be less than one-thirtieth of the dose obtained from the same activity (in mc.) of I^{131} (7, 8). With the gamma-ray detectors currently used in clinical work, approximately 0.7 μc. of I^{132} will yield counts of the magnitude of those from 1 μc. of I^{131} (6, 8).

In the I^{131} treatment of a hyperthyroid patient in whose thyroid gland I^{131} has an effective half-life of, say, five days, 75 per cent of the radiation dose will be delivered to the thyroid gland within ten days. It is to be expected, therefore, that any decrease in the thyroidal radioiodine uptake will be detectable three weeks after a dose of I^{131}; this is also in agreement with the results of other investigations (1, 2, 9, 10). The recovery from a depressed thyroidal function between three weeks and two months after I^{131} therapy, observed in patients requiring retreatment, may be due to any of the following factors: 1) at least partial reversibility of the radiation damage; 2) hyperplasia of the remaining thyroid tissue; 3) the existence of thyroid parenchymal reserves which begin to function when the most active parts have been damaged by irradiation; or 4) compensation of the radiation damage by increased hypophysial thyrotropic stimulation of the persistent thyroidal tissue. It has been shown that even in hyperthyroidism the thyroidal uptake of radioiodine can be further stimulated by administration of exogenous thyrotropin (6, 11).

Reversible radiation damage probably accounts for some at least of the observed "recurrences." Our knowledge of the mechanism of hyperthyroidism is still too imperfect to show whether the other alternatives are conceivable.

Remission is unlikely in a patient in whom the thyroidal radioiodine uptake has not decreased within three weeks after I^{131} treatment (1). It occurred in only 1 of the 20 patients in the present series (*cf.* 10). The prognostic value of this observation is lessened, however, by the fact that more or less pronounced depression of the uptake was also observed in 17 of the 21 patients who required re-treatment. On the average, the reduction of thyroidal radioiodine uptake was less in patients requiring re-treatment, but no attempt to graduate the magnitude of the reduction showed satisfactory accord with the clinical course at the time of follow-up examination. In this fairly small series no patient who exhibited a remission at the time of follow-up had an *H* value exceeding 10 per cent three weeks after treatment (Table 1), whereas half of the patients requiring re-treatment had *H* values exceeding 10 per cent (range, 10.9 to 24.0 per cent) three weeks after treatment.

The indications for radioiodine therapy should not be based on the results of a laboratory test, either initally or three weeks or two months after treatment. However, in severe thyrotoxicosis or serious complicating diseases in which it is difficult for the patients to endure prolonged hyperthyroidism following unsuccessful I^{131} therapy, the interval between therapeutic doses of I^{131} can sometimes be shortened by determination of early radiation effects on the thyroid gland. It is unlikely that re-treatment will be undertaken unnecessarily if the uptake has not decreased three weeks after therapy or—assuming that the present method has been used—if *H* exceeds 10 per cent.

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DRUG-INDUCED LACTATION IN UREMIA

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ABSTRACT

Nonpuerperal lactation occurred in 3 patients with uremia, while they were receiving small amounts of reserpine or phenothiazine derivatives. It is suggested that uremia may facilitate the development of galactorrhea in response to these substances.

ABNORMAL lactation in the nonpregnant female has been observed in a variety of conditions (1). Such galactorrhea may be produced not only by hormonal and central nervous system disturbances but also by extrinsic factors. Recently both reserpine and the phenothiazines have been added to these exogenous causes. This is exemplified by the following 3 uremic patients in whom lactation was associated with the administration of small amounts of reserpine or phenothiazine derivatives.

CASE REPORTS

Case 1

A 23-year-old nulliparous white female was admitted to the hospital because of increasing nausea, anorexia, weight loss and amenorrhea of six months' duration. For two weeks prior to admission she had had nocturia, difficulty in sleeping and pruritis of increasing severity. Her past history revealed recurrent attacks of acute pyelonephritis. There was no history of lactation.

Physical examination upon admission showed a chronically-ill patient with generalized skin pigmentation. There was no discharge from her small, nontender breasts. The blood pressure was 140/90 mm. Hg and there was a loud systolic murmur present over the apex of the heart. The urine was acid with a fixed specific gravity of 1.010; it contained protein (3-plus), and there were numerous white blood cells and clumps in the sediment. Blood chemical studies revealed marked nitrogen retention, the urea nitrogen level being 180 mg., and the creatinine level 15 mg. per 100 ml. Although the concentration of serum calcium was 9 mg. per 100 ml., and of inorganic phosphorus 7 mg. per 100 ml., she was considered to have secondary hyperparathyroidism. X-ray films of the skull demonstrated small areas of demineralization. The sella turcica was normal. Films of the teeth showed absence of the lamina dura and those of the hands showed subperiosteal bone resorption.

A 24-hour urine specimen contained no estrogens (Allen-Doisy bio-assay method) and less than 48 mouse units of follicle-stimulating hormone (FSH) (Klinefelter method). The 24-hour excretion of 17-ketosteroids was 4.9 mg., and of 17-hydroxycorticosteroids 4 mg.

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Because of the persistence of nausea, vomiting and pruritis, several types of phenothiazine derivatives were administered at different intervals. These included prochlorperazine (Compazine), 15 mg. daily for ten days; trimeprazine (Temaril), 7.5 mg daily for two days; and perphenazine (Trilafon), 12 mg daily for four days. On the fifteenth hospital day, while receiving perphenazine, galactorrhea was first noted. Milk could be expressed with ease from both breasts, though neither was enlarged or tender. Within a week after discontinuance of the phenothiazines the galactorrhea subsided. Her course was one of rapid deterioration. At the time of death she was no longer lactating.

The postmortem examination demonstrated acute and chronic pyelonephritis with multiple renal cortical abscesses. The uterus was very small and showed no signs of pregnancy. Histologic studies of the breasts were not made, but they did not appear abnormal. The ovaries, except for several small inclusion cysts, were normal. All of the parathyroid glands were very hyperplastic. The pars intermedia of the pituitary gland contained a very small, encapsulated cyst with a cuboidal epithelial lining. The remainder of the pituitary was not remarkable. The brain showed no changes other than generalized edema.

Case 2

A 29-year-old colored female was known to have hypertension. When cardiac decompensation developed, she was given digitalis, chlorthiazide and 0.75 mg. of reserpine daily for three weeks. At this time, because of progressive oliguria associated with nausea, vomiting and muscular twitchings, she was hospitalized and then transferred to The Jewish Hospital of Brooklyn for consideration of artificial hemodialysis. Her past history included 4 pregnancies, the last one having occurred more than four years prior to the present hospital entry. No information could be obtained concerning recent menstruation or lactation.

Upon admission, the pertinent physical findings were as follows: She was somnolent and her blood pressure was 250/110 mm. Hg. Funduscopic examination showed numerous hemorrhages, exudates and papilledema. Pulmonary congestion, hepatomegaly and peripheral edema were also present. There was a milky discharge from both nipples; it occurred spontaneously without manual expression. The breasts, however, were not engorged or tender. The initial blood urea nitrogen level was 120 mg. per 100 ml., which later increased to 320 mg. The level of creatinine was 17 mg., and of uric acid 21 mg. per 100 ml.

Despite discontinuance of the reserpine, the galactorrhea persisted. However, her regimen now included 30 mg. of prochlorperazine daily for the control of nausea and vomiting. Her illness was progressive and she died nine days following admission.

The postmortem examination revealed generalized arteriolar sclerosis. There was marked involvement of the small arteries and arterioles, particularly those of the pancreas and kidneys. Except for mild congestion of the vessels, the pituitary gland was normal. The vessels of the brain were also congested and showed marked sclerosis with thickened walls. The breasts were not examined histologically. The uterus showed no evidence of pregnancy.

Case 3

A 33-year-old multiparous white female was admitted to the hospital because of severe respiratory distress. She had had pyelonephritis during childhood, and it had recurred at the age of 22 during her first pregnancy. At that time, in addition to proteinuria and pyuria, she had mild hypertension. Her second pregnancy, six years later, was complicated by toxemia and was terminated by cesarian section. Since then she

had not menstruated. Following her second pregnancy, renal insufficiency developed; this was complicated by persistent and severe hypertension and congestive heart failure.

Physical examination revealed a somnolent, pallid, chronically-ill female. The breasts were normal in size, nontender, and free of nodules. An x-ray film of the chest showed marked central congestion, although her lungs were clear to auscultation.

In an attempt to control the hypertension, she had been taking 0.75 mg. of reserpine daily for four years and had not lactated. Upon admission to the hospital the reserpine was discontinued. During the first fifteen days of her hospital stay, she was given only 75 mg. of prochlorperazine to control nausea. Lactation was now noted for the first time. With moderate pressure, milk could be expressed from both nipples. The only other medication administered was a sodium carboxylic resin to control hyperpotassemia.

Chemical studies of her blood showed marked nitrogen retention, the concentration of urea nitrogen being 170 mg per 100 ml. The serum calcium level was 7 mg., and the inorganic phosphorus level 10 mg. per 100 ml. X-ray films of her clavicles demonstrated the typical findings of hyperparathyroidism.

The urine contained no estrogens (Allen-Doisy method) and less than 5 mouse units of FSH in twenty-four hours (kaolin absorption method). The 24-hour excretion of 17-ketosteroids was 3.6 mg., and of 17-hydroxycorticosteroids 1.5 mg.

Although the respiratory distress and uremic symptoms subsided following peritoneal dialysis, these later recurred and the patient died. Consent for postmortem examination was not obtained.

DISCUSSION

Galactorrhea produced by phenothiazine derivatives (2–7) or reserpine (8–10) is being reported with increasing frequency. This phenomenon has been noted in psychiatric as well as in hypertensive, premenopausal patients who received large doses of these drugs (9). The increased incidence may, in part, be related to the occasional spontaneous development of galactorrhea in psychiatric disorders (1) and to the greater diligence with which cases have been sought for by interested observers (6–9).

The mode of action of reserpine or the phenothiazines in the production of lactation is uncertain. These agents, perhaps by antagonizing the activity of serotonin directly, or by diminishing the capacity of cells to bind serotonin, may impair hypothalamic function (1–10). This may explain the observed interference of reserpine or the phenothiazines with hypothalamic regulation of anterior pituitary functions. Such alteration results in the release of certain tropic hormones (3, 11, 12). An increase in the prolactin content of the pituitary has been observed following the injection of reserpine into rabbits (13), and increased urinary excretion of FSH has been reported in patients with reserpine-induced (1) or phenothiazine-induced (3) lactation. However, in 2 of the patients reported here, in whom these determinations were performed, there was a decrease rather than an increase. Such variations in tropic hormone excretion have been noted by others (3). No specific histologic changes in the brain or pituitary gland have been recorded in lactating patients or in experi-

mental animals to whom such drugs were administered. In Case 1 of this study, the small cyst in the pars intermedia of the hypophysis was lined with cuboidal epithelium, and was considered to be non-secretory. The contiguous cells showed no evidence of local pressure.

The phenothiazine derivatives and reserpine are commonly used to alleviate many of the distressing signs and symptoms of uremia such as nausea, vomiting, pruritis and hiccough. They are also used to control hyperirritability and agitation of neuropsychiatric origin, as well as severe hypertension with its concomitant encephalopathy. Such complications of uremia were present in the cases reported here and galactorrhea developed in all 3 patients during treatment with reserpine or phenothiazine derivatives. However, much smaller quantities of the drugs were used in comparison to those which produce lactation in nonuremic patients (4, 9). The metabolic disturbances of the nervous system in uremia, as reflected by diminished cerebral uptake of oxygen and decreased glucose consumption (14, 15) may have made the central nervous system more sensitive to these agents.

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Letters to the Editor

FURTHER OBSERVATIONS ON THE EARLY METABOLIC EFFECTS OF THYROTROPIC HORMONE (HOG PREPARATION)

To the Editor:

The early metabolic effects of thyrotropic hormone (TSH) following its intramuscular administration in man have been described (1, 2). These significant rises in metabolic rate, pulse rate and pulse pressure could not be reproduced during the administration of either triiodothyronine (T_3), thyroxine (T_4) or desiccated thyroid (1–3). There was no rise in metabolic rate with T_4, a slight rise with desiccated thyroid and a greater rise with T_3; the latter increment, however, was significantly less than that following TSH. Further, there appeared to be a qualitative difference between the effects of these hormones and the effect of TSH, in that a rise in urinary inorganic phosphate followed the administration of the desiccated thyroid and triiodothyronine but there was no such rise following administration of TSH.[1]

It was thought desirable to determine the early metabolic effects of a different preparation of TSH from a different animal source (hog pituitaries[2]) and to compare the results with those produced by a heat-inactivated TSH preparation.

METHODS

The experimental methods were essentially the same as those described in previous reports (1, 3). Two groups of subjects were used: 1) a euthyroid group consisting of 5 healthy volunteer male students, and 2) a hypothyroid group consisting of 3 women with postoperative myxedema (subtotal thyroidectomy), who were being maintained in a euthyroid clinical state with adequate doses of desiccated thyroid.

The euthyroid group received physiologic sodium chloride solution (2 ml.) intramuscularly on the first day of observation. TSH was administered intramuscularly one week later (second day of observation) in a dosage of 0.44 unit per Kg., and inactivated TSH was given three weeks after that (third day of observation). Inactivation was achieved by maintaining the TSH at 60° C. for thirty minutes at pH 4.0, following which the pH was adjusted to 5.4 and the preparation autoclaved. The hypothyroid group received physiologic saline the first day, and TSH on two subsequent observation days one week and four weeks respectively after the first day.

[1] Thytropar (Armour Laboratories), prepared from bovine pituitaries.

[2] Primothyron, kindly supplied by Schering, A. G., Berlin.

Observations were made at two-hourly periods for eight hours on the clinical state, pulse rate, pulse pressure, metabolic rate, plasma protein-bound iodine (PBI) level (4), plasma and urinary inorganic phosphate levels (5), and urinary creatinine output (6).

RESULTS

Normal subjects receiving TSH complained of irritation and pain at the site of injection, and also nausea and headache lasting for several hours. No local effects in the thyroid area, such as pain or swelling, were noticed. These symptoms were less evident in the hypothyroid group given TSH,

TABLE 1. EFFECT OF HOG TSH ON METABOLIC RATE (CALS./SQ. M./HR.)

Treatment	Time in hours			
	1	3	5	7
*Normal Subjects (mean of 5)**				
Physiologic saline	49.0	48.5	48.1	48.3
TSH	47.1	50.5	52.7	55.0
Inactivated TSH	47.3	47.1	48.2	49.1
Myxedematous Subjects Maintained with Desicc. Thyroid (mean of 3)†				
Physiologic saline	47.2	45.3	45.8	47.3
TSH—1	45.7	49.4	49.4	49.6
TSH—2	43.2	44.0	45.6	46.1

* Trend analysis showed a significantly greater rise following TSH when compared with saline and inactive TSH ($P < 0.001$).

† Trend analysis showed a significant increase following TSH on both days compared with the control ($P < 0.05$). However, the rise with TSH was significantly less than that observed in normal subjects ($P < 0.01$).

and absent in the euthyroid group following administration of inactive TSH.

A rapid rise in the metabolic rate was observed in the normal subjects following injection of TSH (Table 1), and trend analysis revealed that the rise was significantly higher than that induced by physiologic saline ($P < 0.001$). Following administration of heat-inactivated TSH, there was no significant rise in metabolic rate. In the hypothyroid group there was a small rise in metabolic rate following TSH on both days; this reached significance, according to the trend analysis, although some of the effect was related to the high initial level of the metabolic rate on the control day. However, comparison of the trends in metabolic rate following TSH in the hypothyroid subjects on both days showed that the rise was significantly less than that for the euthyroid goup.

The effects on pulse rate and pulse pressure in the normal subjects paralleled closely the effects on the metabolic rate (Table 2). No significant effects on pulse rate or pulse pressure were noted following administration of TSH in the myxedematous group.

TABLE 2. EFFECT OF HOG TSH ON PULSE RATE AND PULSE PRESSURE IN NORMAL SUBJECTS

Treatment	Time in hours				
	0	2	4	6	8
Pulse Rate (mean for 5 subjects)					
Physiologic saline	69	62	60	63	64
TSH	65	66	77**	71**	76**
Inactivated TSH	62	59	62	59	59
Pulse Pressure (mm. Hg) (mean for 5 subjects)					
Physiologic saline	45	48	41	41	47
TSH	43	49	52**	54**	51
Inactivated TSH	45	43	44	44	43

** $P < 0.01$ compared with that for saline at same time, by analysis of variance.

The effects on the concentration of plasma PBI are recorded in Table 3. The data demonstrate that the TSH preparation was substantially inactivated by the process described, and that the hypothyroid group had very little residual functioning thyroid tissue.

In the normal subjects there was no rise in the level of plasma inorganic phosphate following administration of active TSH, nor were there any consistent effects on the urinary excretion of inorganic phosphate.

DISCUSSION

The results reported here on the early metabolic effects of a preparation of hog TSH confirm those reported by Hetzel *et al.* (1, 2) on the effects of bovine TSH. There was an early increase in metabolic rate following

TABLE 3. EFFECT OF HOG TSH ON PLASMA PBI LEVEL (μG./100 ML.)

Treatment	Time in hours		
	0	4	8
Normal Subjects (mean of 5)			
Physiologic saline	4.6	4.4	4.6
TSH	4.6	6.1**	7.8***
Inactivated TSH	4.8	5.4*	5.4*
Myxedematous Subjects (mean of 3)			
Physiologic saline	5.4	5.3	5.1
TSH—1	4.9	4.7	4.8
TSH—2	4.1	4.3	4.2

* $P < 0.05$
** $P < 0.01$ } Compared with that for control at same time, by analysis of variance.
*** $P < 0.001$

The increase with active TSH was greater than that with inactive TSH at 4 hours ($P < 0.01$) and 8 hours ($P < 0.001$).

administration of TSH, which effect was nullified when the TSH was inactivated. The effect was also significantly reduced when TSH was given to thyroid-maintained hypothyroid subjects.

These data confirm the previous finding that the early metabolic effect of TSH is primarily mediated through an intact thyroid gland and is not merely due to a nonspecific response to parenteral protein. This hog preparation is stated to be free from posterior pituitary factors, and follicle-stimulating, adrenocorticotropic and luteotropic hormones. The most likely contaminant is luteotropic hormone (7), but this agent has been administered to hypophysectomized rats without a subsequent rise in metabolic rate (8).

The possible mechanisms of this early rise in metabolic rate after administration of TSH—a rise which has not been reproduced after administration of known thyroid hormones—have been discussed previously (2, 3). "Thyroiditis-like" reactions have more recently been reported (9), but it is unlikely that this is the cause of the increase in general metabolism, as such reactions were absent in our group of normal subjects. We have, however, subsequently observed these effects in 2 of 5 euthyroid subjects when given hog TSH from another batch.

Another possibility, previously suggested, is that TSH stimulates the secretion of a rapidly-acting thyroid hormone distinct from those already known (1–3). This hypothesis can be further studied only by employing chromatographic methods, and such investigations are in progress in this laboratory.

SUMMARY

Early metabolic effects similar to those previously reported for a bovine TSH preparation have now been observed following administration of a hog TSH preparation. These effects were nullified by inactivation of the TSH.

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May 4, 1961

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EFFECT OF LEUCINE ON PLASMA INSULIN CONCENTRATION IN A GIRL WITH A PANCREATIC ADENOMA

To the Editor:

The hypoglycemia following ingestion of leucine in certain patients with idiopathic hypoglycemia is associated with an increased concentration of plasma insulin (1–3).

Recently leucine-induced hypoglycemia has been observed in cases of pancreatic islet-cell tumor (4). Flanagan *et al.* suggest that leucine acts in these patients "by potentiating the peripheral action of insulin" (4). However, these authors realized that increased secretion of insulin after leucine ingestion had not been ruled out. This has prompted us to report the results of insulin assays in an 11-year-old girl with a pancreatic adenoma. The assays were performed immunologically by Dr. R. S. Yalow and Dr. S. A. Berson, who also performed assays on the sera of patients with leucine-sensitive idiopathic hypoglycemia (1–3).

CASE REPORT

An 11-year-old otherwise healthy girl had suffered from attacks of hypoglycemia for eight months. The attacks, manifested by weakness often associated with convulsions,

always occurred before breakfast. Her mother noted increased irritability and emotional lability just prior to other meals as well.

On October 25, 1960, an islet-cell adenoma was excised from the head of the pancreas by Dr. W. K. Sieber. The tumor was encapsulated and weighed 1.8 gm. The postopera-

TABLE 1. EFFECT OF LEUCINE ON BLOOD SUGAR AND PLASMA INSULIN LEVELS, BEFORE AND AFTER OPERATION

	Blood sugar level (mg./100 ml.)			
	Before operation*		After operation*	
	Oct. 17	Oct. 18	Nov. 1	Nov. 30
Fasting	41	51	100	103
Leucine†				
15 mins.	26	35	104	96
30 mins.	14	49	108	79
1 hr.	22	52	104	86
2 hrs.	42	42	102	114
	Plasma insulin level (μU/ml.)			
	Oct. 18		Nov. 30	
Fasting	100		74	
Leucine†				
1 hr.	265		95	

* Tumor removed October 25, 1960.
† Leucine ingested in casein hydrolysate, 1.5 gm./kg. body weight.

tive course was uneventful, and there has been no recurrence of signs or symptoms in the ensuing seven months. Since the operation, the blood sugar level, even after periods of fasting up to eighteen hours, has always been in the normal range.

The blood sugar responses to leucine ingestion on two occasions before operation and on two occasions afterward, are recorded in Table 1. Blood was obtained for insulin assay during the second of each pair of leucine tolerance tests. These results are also recorded in the table. Leucine was provided by casein hydrolysate ingested in doses of 1.5 gm. per kg. of body weight on each occasion.

The data show that before operation, when the patient was fasting, the plasma insulin level was abnormally elevated. Plasma insulin concentration increased significantly following ingestion of leucine even though the blood specimen for assay was not obtained at the time of maximal hypoglycemia. After operation, with the patient fasting, the plasma insulin level was within normal limits. The significance of the slight fall in blood sugar concentration during the last leucine tolerance test and of a plasma insulin level (fasting) close to the upper limit of normal, is not clear. The patient is being kept under observation in view of the possibility of recurrence of the tumor.

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April 24, 1961

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A NEW METHOD FOR THE DETERMINATION OF URINARY AROMATIC ACIDS BY GAS CHROMATOGRAPHY

To the Editor:

We have described recently a simple method for the separation of the urinary aromatic acids by gas-liquid partition chromatography (1). By this method, at least 40 peaks have been detected consistently in the chromatogram of normal human urine. Of these, 6 peaks have been related to known urinary metabolites by comparing their retention times with those of standards on two different columns. In this paper we wish to illustrate the ease with which an abnormal excretion of a particular metabolite such as 5-hydroxyindoleacetic acid can be detected and determined quantitatively.

CASE REPORT

R.G.D., a 35-year-old white male, was admitted to the Veterans Administration Hospital with a sixteen-year history of paroxysmal attacks of shortness of breath associated with flushing of the skin. These attacks occurred daily, were precipitated by exertion and excitement, and were accompanied by a feeling of warmth and perspiration over the entire body surface. During the previous three to four years, the patient noticed that his face had become permanently discolored a purplish-blue.

On physical examination, malar cyanosis and a generalized facial flush were obvious. Marked wheezing was heard throughout both lung fields, and a grade 2 systolic murmur in the tricuspid area. A tender mass was palpated in the right lower quadrant; when massaged, the patient experienced a characteristic flush. A screening test for 5-hydroxyindoleacetic acid yielded positive results. At laparotomy an unresectable 7-cm. lesion was found imbedded in the mesentery of the small bowel. Associated metastatic nodules were seen over the entire liver; one of them was taken for a biopsy specimen. The pathologist reported it to be carcinoid.

METHODS AND RESULTS

A morning specimen of urine was collected from this patient on the

sixth postoperative day. The specimen was acidified and assayed for creatinine; the urinary aromatic acids were then extracted from an aliquot containing 20 mg. of creatinine, by the method of Shaw *et al.* (2). The final ethyl acetate extract was reduced to dryness under a stream of nitrogen at room temperature and methylated with diazomethane as described elsewhere (1). The reaction mixture was evaporated under a

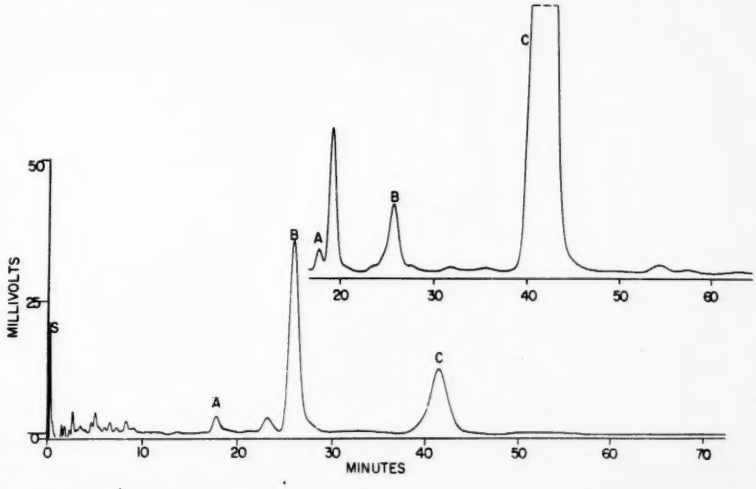

FIG. 1. Gas chromatographic analysis on adipate polyester column at 206° C. of methyl esters of aromatic acids from the urine of a patient with carcinoid (aliquot equivalent, 400 μg. of creatinine). The insert at the upper right is the graph for urine from a normal subject (aliquot equivalent, 400 μg. of creatinine). S = solvent; A = homovanillic acid; B = p-hydroxyphenylacetic acid; C = hippuric acid.

stream of nitrogen to remove unused diazomethane, and the residue of the methylated esters of the aromatic acids was taken up in 250 μl. of ethyl ether. Five-microliter samples (corresponding to a volume of urine containing 400 μg. of creatinine) were analyzed by gas chromatography in the Barber-Colman Model 10 instrument. The results are shown in Figures 1 and 2, illustrating the profile of the aromatic acids in the urine of the patient with carcinoid; the inserts show comparable areas of profiles of the aromatic acids in the urine of normal subjects.

Urine samples from 4 apparently healthy subjects were analyzed in a similar manner and quantitative measurements of 5 aromatic acids were made from these data. Pure reference samples of aromatic acids were methylated with diazomethane, and analyses were made of each sample with concentrations ranging from 1 to 15 μg. Quantitative estimates of urinary excretion were made by comparing the area of a particular peak

with a plot of the area versus the concentration of that component. The standard curves were linear for the concentration range found in the urine. The low concentration of 5-hydroxyindoleacetic acid (Fig. 2, insert, component F) required an increase in sensitivity (×3.3) of the instrument for accurate measurement. A tenfold reduction of sample size was necessary in order to measure the hippuric acid peak in analyses of normal urine.

The results are summarized in Table 1, together with previously reported values in the literature. For compounds with values reported in the literature, the comparisons are fairly good. The values for 5-hydroxyindolacetic

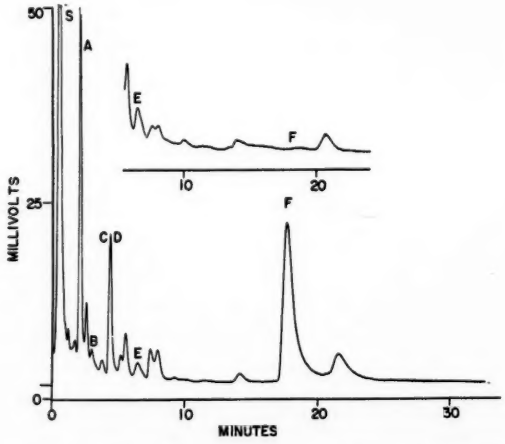

FIG. 2. Gas chromatographic analysis on silicone column at 191° C. of methyl esters of aromatic acids from the urine of a patient with carcinoid (aliquot equivalent, 80 μg. of creatinine). The insert at the top is the graph for urine from a normal subject (aliquot equivalent, 80 μg. of creatinine). S = solvent; A = p-hydroxyphenylacetic acid; B = homovanillic acid; C = hippuric acid; D = 3-methoxy-4-hydroxymandelic acid; E = indoleacetic acid; F = 5-hydroxyindoleacetic acid.

acid were somewhat lower than those reported by Armstrong *et al.* (4) who used a paper chromatographic method, or by Olson *et al.* (5) who used the method of Udenfriend *et al.* (6). It may be that the colorimetric method of Udenfriend is not entirely specific for 5-hydroxyindoleacetic acid. The excretion of this metabolite by our patient with malignant carcinoid was more than twenty times greater than the highest value recorded for the controls—an order of magnitude similar to that reported by others. The excretion of indoleacetic acid by this patient was at the lower limits of the normal range. This may be analogous to the diminished excretion of N-methylnicotinamide by patients with carcinoid, as reported by Sjoerdsma *et al.* (7), and may be related to the abnormal conversion of the

TABLE 1. URINARY AROMATIC AND METHYLATED PHENOLIC ACIDS
(μg./mg. creatinine)

Compound	In urine from patient with carcinoid	In urine from 4 normal adults (mean & range)	Values reported in the literature* (mean or range & reference)
P-Hydroxyphenylacetic acid	78	19 [8 to 42]	— —
Homovanillic acid	2.5	3.6 [2.6 to 4.8]	— [2.1 to 5.7] (2)
Hippuric acid	51	660 [320 to 830]	500 (3)
5-Hydroxyindoleacetic acid	29	1.1 [0.8 to 1.3]	— [1 to 3] (4)
Indoleacetic acid	0.8	1.4 [0.8 to 2.2]	— [<1 to 10] (4)

* Values for homovanillic acid and hippuric acid were calculated by assuming an average creatinine excretion of 1.4 gm. per day.

5-hydroxytryptamine pathway from a minor (1 per cent) to a major (60 per cent) route of tryptophan metabolism, thus depriving the tryptamine and kynurenine pathways of normal substrate levels. Homovanillic acid excretion by the carcinoid patient and the group of controls was within the limits of the normal range reported by Shaw *et al.* (2). Hippuric acid excretion was reduced tenfold in the patient with carcinoid; this may reflect an over-all reduction in liver function as a result of the extensive metastases or, alternatively, it may indicate a decrease in the amount of benzoic acid reaching the liver for inactivation. Urinary p-hydroxyphenylacetic acid represents a minor pathway of tyrosine metabolism, accounting for about 1 per cent of dietary tyrosine (8). The high value recorded for the patient is probably due to the fact that a high-protein diet had been started several days prior to the urinalysis.

No particular significance can be attached to the changes in excretion of aromatic acid metabolites observed on this one occasion in one patient, since he had been receiving hydrocortisone (150 mg. of Solu-Cortef, Upjohn) daily for two weeks prior to the collection. However it is obvious that this method is well suited for the systematic study of aberrations in metabolism of the various related aromatic amines produced in particular disease states or by particular endocrine factors. In addition, it may well replace more tedious and less specific methods for the quantitative determination of such diagnostically significant aromatic acids as 3-methoxy-4-hydroxymandelic acid in pheochromocytoma and neuroblastoma (9), phenylpyruvic acid and *o*-hydroxyphenylacetic acid (10) in phenylketonuria, homogentisic acid in alkaptonuria, indoleacetic acid in Hartnup

disease, and *p*-hydroxyphenylpyruvic acid in tyrosinosis, as well as 5-hydroxyindoleacetic acid in malignant carcinoid.

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The Endocrine Society

The 1962 Annual Meeting

The Forty-Fourth Annual Meeting of The Endocrine Society will be held in the Palmer House, Chicago, Illinois, Thursday, Friday and Saturday, *June 21–22–23, 1962.*

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 22, at 7:30 P.M. preceded by cocktails at 6:30 P.M.

The Chairman of Local Arrangements is Dr. Allan T. Kenyon.

All members are urged to make their hotel reservations immediately. The Palmer House will hold 300 bedrooms for members until May 1, 1962, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining for the A.M.A. meeting, please advise the hotel. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1962.

Everyone attending the meetings is required to register. By action of the Council, non-members will be charged a registration fee of $15.00 for any part of the scientific sessions. Members will be charged a registration fee of $5.00.

Graduate students, residents and interns, and members of the medical departments of the armed forces will be admitted upon presentation of proper credentials CERTIFIED BY DEPARTMENT HEAD OR SUPERINTENDENT OF HOSPITAL and payment of $5.00 registration fee.

Final program, membership card and advance registration forms will be sent on May 15, 1962, to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Leslie L. Bennett, Dept. of Physiology, University of California Medical Center, San Francisco 22, California, *not later than February 15, 1962.* It is imperative that the abstracts be informa-

tive and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas, graphs or extensive tables cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2 Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5″ filing cards, one for each author, giving his name and initials in the upper right hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors and the mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Bennett.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

The 1962 Awards

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors. Endocrine Society awards are limited to citizens of the United States and Canada.

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle and in 1960 was presented to Dr. Emil Witschi. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954.

The Ciba Award

The Ciba Award is given in recognition of the meritorious accomplishments of an investigator in the field of basic or clinical endocrinology who has not reached his thirty-sixth birthday before June 1 of the year in which the Award is presented. This Award was established in 1942 and first presented in 1944. The Award is presently in the amount of $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $7,500, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Awards

The Council of The Endocrine Society has approved a change in status of the Upjohn Scholar to that of The Upjohn Awards.

There will be two awards in the amount of $1,250 each, one for the best article in format and content printed in Endocrinology and one in The Journal of Clinical Endocrinology and Metabolism during the preceding year.

Awardees will be chosen by the Awards Committee at its regular meeting in November of each year.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

Purified Radioactive Steroids Available

The Endocrinology Study Section, Division of Research Grants, National Institutes of Health, has the following purified radioactive steroids available for distribution to qualified investigators, free of charge except for $5.00 per ampule to cover handling and shipping. The materials are stored at Tracerlab, Inc., Waltham, Massachusetts, and the recipient will be billed by Tracerlab at the rate of $5.00 per ampule.

Material	*Batch*	*Specific Activity*
Hydrocortisone 4-C^{14}	ES-F-10-1	8.7 μc./mg.
Hydrocortisone 4-C^{14}	ES-F-50-1	42.4 μc./mg.
Corticosterone 4-C^{14}	ES-B-25-1	22.8 μc./mg.
Corticosterone 4-C^{14}	ES-B-50-1	44.4 μc./mg.
7-^{3}H aldosterone		20.0 μc./μg.
7-^{3}H cortisol		28.0 μc./μg.

Investigators may obtain supplies of these materials by sending a brief description of the proposed usage, together with an estimate of the amount of materials required. Requests are filled as promptly as possible after a review by the Committee on Radioactive Hormone Distribution.

The 4-C^{14} compounds are sealed in ampules containing 5 μc., 10 μc., or 25 μc. each, in a solution of 95% benzene and 5% methanol, and are about 96% pure. The 7-^{3}H steroids are also in a solution of benzene and methanol, the aldosterone sealed in 60-μc. ampules, and the cortisol in 30-μc. ampules. Investigators are cautioned to calibrate each batch of material before use.

The applicant's Atomic Energy Commission registration number governing the use of isotopic materials should accompany all requests. In addition, a voucher number or purchase order certifying that the handling charges will be paid to Tracerlab, Inc., together with the exact address of the applicant, would be appreciated. This will avoid loss or undue delay, and will assist Tracerlab in collecting the handling charge due them for processing materials.

Requests for these compounds should be submitted to: Morris M. Graff, Executive Secretary, Endocrinology Study Section, Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland.

Purified Pituitary Hormones Available

The Endocrinology Study Section, Division of Research Grants, National Institutes of Health, has the following highly purified pituitary hormones available for distribution free to qualified investigators:

Growth Hormone, bovine, non-sterile, for animal experiments only. Bulk; dispensed as requested.

Follicle-stimulating hormone, ovine, sterile preparation; 25 mg. vials for experiment; 5 mg. vials for assay standard.
Prolactin, ovine, sterile preparation; 25 mg. vials.

Investigators may obtain supplies of these hormones by sending a brief description of the proposed usage, together with an estimate of the amount of materials required. Requests are filled as promptly as possible from supplies maintained by Dr. A. E. Wilhelmi of Emory University, Atlanta, Georgia, after review by the Committee on Hormone Distribution. These hormones are prepared and distributed under a program generously supported by the National Institutes of Health.

Requests for these materials should be submitted to: Morris M. Graff, Executive Secretary, Endocrinology Study Section, Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland.